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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)		37690-II-PCT-US

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US92/01143	10 February 1992	08 February 1991

TITLE OF INVENTION
CD4 GAMMA2 AND CD4-IgG2 CHIMERAS

APPLICANT(S) FOR DO/EO/US
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Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C. 371:

1. ☒ This express request to immediately begin national examination procedures (35 U.S.C. 371(f)).
2. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
	TOTAL CLAIMS	14 -20=	0	X$ 22.00	$ 0
	INDEPENDENT CLAIMS	4 -3=	1	X$ 74.00	74.00
	MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+$ 130.00	130.00
	BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)): ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... $640 ☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445 (a)(2)) ..... $710 ☒ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... $950 ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4)..... $ 90				950.00
	Surcharge of $120 for furnishing the National fee or oath or declaration later than ☒20 ☐ 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).				130.00
			TOTAL OF ABOVE CALCULATIONS		= 1284.00
	Reduction by 1/2 for filing by small entity, if applicable. Affidavit musts be filed also. (Note 37 CFR 1.9, 1.27, 1.28.)				642.00
			SUBTOTAL		+ 642.00
	Processing fee of $30 for furnishing the English Translation later than ☐20 ☐30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).				0
			TOTAL NATIONAL FEE		$ 642.00
	Fee for recording the enclosed assignment (37 CFR 1.21(h)).				+ 40.00
			TOTAL FEES ENCLOSED		$ 682.00

a. ☒ A check in the amount of $ 682.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 03-3125. A duplicate copy of this sheet is enclosed.

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3. A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
   c. ☐ has been transmitted by the International Bureau.
4. ☐ A translation of the International Application into English (35 U.S.C. 371 (c)(2)).
5. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
6. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
7. ☒ An oath or declaration of the inventor (35 U.S.C. 371(c)(4)).
8. ☐ A translation of the Annexes to the International Preliminary Examination Report under PCT Article 36(35 U.S.C. 371(c)(5)).

Other document(s) or information included:

9. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
10. ☒ An assignment document for recording.
    Please mail the recorded assignment document to:
    a. ☒ the person whose signature, name & address appears at the bottom of this page.
    b. ☐ the following:

X Preliminary Amendment

X Verified Statement Claiming Small Entity Status Under 37 C.F.R. sl.9(f) and sl.27(c) - Small Business Concern

11. The above checked items are being transmitted
    a. ☐ before the 18th month publication.
    b. ☐ after publication and the Article 20 communication but before 20 months from the priority date.
    c. ☒ after 20 months but before 22 months (surcharge and/or processing fee included).
    d. ☐ after 22 months (surcharge and/or processing fee included).
       Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted after 22 months *and no proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.*
    e. ☐ by 30 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
    f. ☐ after 30 months but before 32 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date (surcharge and/or processing fee included).
    g. ☐ after 32 months (surcharge and/or processing fee included).
       Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted after 32 months and a proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.
12. At the time of transmittal, the time limit for amending claims under Article 19
    a. ☒ has expired and no amendments were made.
    b. ☐ has not yet expired.
13. ☐ Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____ (date), namely:

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28,678
REGISTRATION NUMBER

Form PTO-1390 (Rev. 8-87) USCOMM-DC-87-3805

CD4-GAMMA2 AND CD4-IgG2 CHIMERAS

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The life cycle of animal viruses is characterized by a series of events that are required for the productive infection of the host cell. The initial step in the replicative cycle is the attachment of the virus to the cell surface which is mediated by the specific interaction of the viral attachment protein (VAP) to receptors on the surface of the target cell. The pattern of expression of these receptors is largely responsible for the host range and tropic properties of viruses. The interaction of the VAP with cellular receptors therefore plays a critical role in infection and pathogenesis of viral diseases and represents an important area to target the development of anti-viral therapeutics.

Cellular receptors may be comprised of all the components of membranes, including proteins, carbohydrates, and lipids. Identification of the molecules mediating the attachment of viruses to the target cell surface has been made in a few instances. The most extensively characterized viral receptor protein is CD4 (T4) (1). CD4 is a nonpolymorphic

cell surface glycoprotein that is expressed primarily on the surface of helper T lymphocytes and cells of the monocyte/macrophage lineage. CD4 associates with major histocompatibility complex (MHC) class II molecules on the surface of antigen-presenting cells to mediate efficient cellular immune response interactions. In man, CD4 is also the target of interaction with the human immunodeficiency virus (HIV).

HIV infects primarily helper T lymphocytes and monocytes/macrophages, cells that express surface CD4, leading to a gradual loss of immune function which results in the development of the human acquired immune deficiency syndrome (AIDS). The initial phase of the HIV replicative cycle involves the high affinity interaction between the HIV exterior envelope glycoprotein gp120 and surface CD4 (Kd approximately 4 x 10^{-9} M) (2). Several lines of evidence demonstrate the requirement of this interaction for viral infectivity. In vitro, the introduction of a functional cDNA encoding CD4 into human cells which do not express CD4 is sufficient to render otherwise resistant cells susceptible to HIV infection (3). In vivo, viral infection appears to be restricted to cells expressing CD4. Following the binding of HIV gp120 to cell surface CD4, viral and target cell membranes fuse, resulting in the introduction of the viral capsid into the target cell cytoplasm.

Characterization of the interaction between HIV gp120 and CD4 has been facilitated by the isolation of cDNA clones encoding both molecules (4, 5). CD4 is a nonpolymorphic, lineage-restricted cell surface glycoprotein that is a member of the immunoglobulin gene superfamily. High-level expression of both full-length CD4 and truncated, soluble versions of CD4 (sCD4) have been described in stable expression systems. The availability of large quantities of purified sCD4 has permitted a detailed understanding of the structure of this complex glycoprotein. Mature CD4 has a relative molecular mass (Mr) of 55 kilodaltons and consists

of an amino-terminal 372 amino acid extracellular domain containing four tandem immunoglobulin-like regions denoted V1-V4, followed by a 23 amino acid transmembrane domain and a 38 amino acid cytoplasmic segment. The amino-terminal immunoglobulin-like domain V1 bears 32% homology with kappa light chain variable domains. Three of the four immunoglobulin-like domains contain a disulphide bond (V1, V2 and V4), and both N-linked glycosylation sites in the carboxy-terminal portion of the molecule are utilized (4, 6).

Experiments using truncated sCD4 proteins demonstrate that the determinants of high-affinity binding to HIV gp120 lie within the amino-terminal immunoglobulin-like domain V1 (7-9). Mutational analysis of V1 has defined a discrete gp120 binding site (residues 38-52 of the mature CD4 protein) that comprises a region structurally homologous to the second complementarity-determining region (CDR2) of immunoglobulins (9). The production of large quantities of V1V2 has permitted a structural analysis of the two amino-terminal immunoglobulin-like domains. The structure determined at 2.3 angstrom resolution reveals that the molecule has two tightly associated domains containing the immunoglobulin-fold connected by a continuous beta strand. The putative binding sites for monoclonal antibodies, class II MHC molecules and HIV gp120 (as determined by mutational analysis) map on the molecular surface (10, 11).

A soluble version of the entire extracellular segment of CD4 (V1-V4, termed sCD4) has been described and appears to be a potential therapeutic approach to the treatment of HIV infection (12). In vitro experiments demonstrate that: 1) SCD4 acts as a "molecular decoy" by binding to HIV gp120 and inhibiting viral attachment to and subsequent infection of human cells; 2) sCD4 "strips" the viral envelope glycoprotein gp120 from the viral surface; and 3) sCD4

blocks the intercellular spread of virus from HIV-infected cells to uninfected cells by inhibiting virus-mediated cell fusion (1, 13).

In addition to in vitro results, experiments with sCD4 in simian immunodeficiency virus (SIV)-infected rhesus monkeys have been described. These studies demonstrated that administration of 2 milligrams (intramuscular) of sCD4 for 28 days to SIV-infected rhesus monkeys led to a decreased ability to isolate virus from peripheral blood lymphocytes and bone marrow. In addition, the growth of granulocyte-macrophage and erythrocyte progenitor colonies in the bone marrow returned to normal levels. These data suggest that administration of sCD4 to SIV-infected rhesus monkeys leads to a diminution of the viral reservoir.

Phase I human clinical trials demonstrated that there is no significant toxicity or immunogenicity associated with administration of sCD4 at doses as high as 30 mg/day. Pharmocokinetic studies revealed the serum half-life of sCD4 to be 45 minutes following intravenous administration, 9.4 hours after intramuscular dosing, and 10.3 hours after the drug was given subcutaneously (14, 15). Preliminary antiviral studies were inconclusive with respect to CD4 cell count and levels of HIV antigen. Because the maximum tolerated dose was not reached, the antiviral effect of sCD4 may have been underestimated, especially in light of recent data concerning differences in sCD4 concentrations required to inhibit laboratory strains of HIV-1 compared to primary viral isolates (16).

Although these in vitro, primate, and human clinical studies with sCD4 have produced encouraging results, they have also defined several limitations. First, the measured serum half-life of sCD4 is relatively short. Second, sCD4 is monovalent with respect to gp120 binding in contrast with

cell surface CD4 and viral surface gp120 which are multivalent. Third, sCD4 is not cytotoxic for HIV-infected cells. Fourth, sCD4 may not cross the placenta to a significant degree. Therefore, chimeric CD4 molecules have been described which take advantage of the immunoglobulin-like nature of CD4 and several beneficial properties of immunoglobulins themselves (i.e. CD4-immunoglobulin fusions).

Immunoglobulins, or antibodies, are the antigen-binding molecules produced by B lymphocytes which comprise the humoral immune response. The basic unit of an immunoglobulin molecule consists of two identical heavy chains and two identical light chains. The amino-terminus of each chain contains a region of variable amino acid sequence (variable region). The variable regions of the heavy and light chains interact to form two antigen binding sites. The carboxy-terminus of each chain contains a region of constant amino acid sequence (constant region). The light chain contains a single constant domain, whereas the heavy chain constant domain is subdivided into four separate domains (CH1, hinge, CH2, and CH3). The heavy chains of immunoglobulin molecules are of several types, including mu (M), delta (D), gamma (G), alpha (A) and epsilon (E). The light chains of immunoglobulin molecules are of two types, either kappa or lambda. Within the individual types of heavy and light chains exist subtypes which may differ in effector function. An assembled immunoglobulin molecule derives its name from the type of heavy chain that it possesses.

The development of monoclonal antibodies has circumvented the inherent heterogeneity of antibodies obtained from serum of animals or humans. However, most monoclonal antibodies are derived from cells of mouse origin and therefore are immunogenic when administered to humans. More recent

developments combining the techniques of molecular genetics with monoclonal antibody technology has lead to the production of "humanized" chimeric antibodies in vitro. In these chimeric antibodies, the variable domains of human immunoglobulin heavy and light chains are replaced with specific heavy and light chain variable domains from a murine monoclonal antibody (17-19). The result of this genetic manipulation is a molecule with specificity for a particular antigen and the characteristics of human immunoglobulins.

Sequence and structural analyses of CD4 indicate that the four extracellular domains are immunoglobulin-like. Since the Fc portion of immunoglobulins controls the rate of catabolism of the molecules (serum half-life ranging from 14 to 21 days) and provides various effector functions, several reports describe the replacement of variable and constant domains of immunoglobulins with the immunoglobulin-like domains of CD4 (21-24).

CD4-IgG1 heavy chain fusion proteins resulting in chimeric gamma1 heavy chain dimers have been described (21). These molecules contain the gamma1 heavy chain CH1 domain in addition to the hinge, CH2 and CH3 domains. However, heavy chain assembly and secretion from mammalian cells is less efficient if the CH1 domain is expressed in the absence of light chains (25). Subsequently, a CD4-IgG1 heavy chain fusion protein lacking the CH1 domain and the first five amino acids of the hinge region was described which was secreted to high levels (22). These fusion proteins retain various effector functions of immunoglobulin molecules, such as Fc receptor binding, antibody-dependent cell-mediated cytotoxicity (ADCC) toward HIV-1-infected cells, and placental transfer via an Fc receptor-dependent mechanism (22). CD4-IgM heavy chain fusion proteins have also been described (26). In addition, CD4-IgG1 fusion proteins have

been described wherein the V1V2 domains of CD4 are fused to the CH1, hinge, CH2 and CH3 domains of a gamma1 heavy chain, and wherein the V1V2 domains of CD4 are fused to the constant domain of a kappa light chain (29).

Fusion proteins linking CD4 to toxins have also been constructed and tested for their ability to kill HIV-infected cells. In one study, sCD4 was coupled to the deglycosylated A chain of ricin which inactivates ribosomes, therefore inhibiting protein synthesis and killing the cell (27). This fusion protein was reported to specifically lyse cells infected with five different isolates of HIV, but was nontoxic to uninfected cells. In another study, the V1V2 domains of CD4 were coupled to domains II and III of Pseudomonas exotoxin A (28). This fusion protein was reported to specifically bind and inhibit protein synthesis in cells expressing the HIV envelope glycoprotein gp120 (25).

It is well established that human monocytes and macrophages (M/M) express surface CD4, can be infected by HIV, and serve as a reservoir of infection and a vehicle for viral dissemination (29). Furthermore human M/M also contain Fc receptors, which are responsible for binding to specific IgG molecules via their Fc portion (see Table 1). The high affinity Fc receptor (FcRI) binds monomeric IgG and complexed IgG (antigen plus antibody). The rank order of affinity of FcRI for IgG isotypes is IgG1=IgG3 > IgG4, and does not interact with IgG2. The low affinity Fc receptor (FcRII) binds monomeric IgG with lower affinity than IgG in complexed form. The rank order of affinity is that IgG1 and IgG3 binding is greater than that of IgG2 or IgG4 (30).

FcReceptor	Molecular Weight	Affinity	Expression	Affinity for isotypes
FcRI	72,000	High	Monocytes	IgG1,IgG3>IgG4, does not bind IgG2
FcRII	40,000	Low	Monocytes, platelets, neutrophils	IgG1,IgG3>IgG2, IgG4
FcRIII	50-70,000	Low	Neutrophils NK, K, monocytes	IgG1,IgG3

(Table abbreviated from Gergely J. and Sarmay G. (1990) FASEB J. 4:3275

Because of the recent demonstration that HIV+ patients' sera contain low titer antibodies which recognize the HIV envelope glycoprotein, it has been observed that infection of M/M is enhanced by low titer anti-HIV antibodies, presumably by cross bridging HIV and the Fc receptor (31). Enhanced infection of macrophages by Dengue virus, Yellow fever virus, and Sindbis virus, is well documented in vitro as well as in Rhesus monkeys (32). Such enhancement has been demonstrated to occur in the presence of subneutralizing antibodies to these viruses, which serves to opsonize the viruses and bind them to the FcRs (or complement receptors) on the surface of the cell. In the case of HIV, this crossbridging serves to concentrate HIV

onto the surface of the M/M, whereupon the virus is then able to utilize CD4 for entry into the cell, since sCD4 is able to inhibit the enhancement seen with low titer antibodies (31).

Recently, Byrn et al. (22) have produced a CD4-IgG chimera of the IgG1 isotype, to increase the plasma half-life of sCD4 as well as to confer effector functions to the chimeric molecule. Therefore this molecule has the potential to bind to Fc receptors located on the surface of the M/M, and potentially cause an increase in the infection of these cell types. Because enhanced infection of these cell types is a serious consideration in developing novel therapeutics, our objective for designing a CD4-IgG molecule was to use the IgG2 type, which has a greatly diminished ability to bind M/M Fc receptors (30). Furthermore, human IgG2 antibodies appear to lack significant allotypic variation, whereas human IgG1 antibodies contain allotypic variations (33). Therefore, to avoid potential immunogenic responses to recombinant molecules containing immunoglobulin domains, we have chosen a molecule which is the least polymorphic and has a decreased ability to concentrate HIV onto the surface of the macrophage.

Second, similar observations of enhanced infection of unborn babies may also be demonstrated for CD4-IgG1 immunoadhesions administered to pregnant mothers. For example, it is well documented that the placental syncytiotrophoblast plasma membrane contains Fc receptors (30). Because materno-fetal transport of immunoglobulin is primarily restricted to the IgG class, it is believed that passive immunity can be achieved by specific transport across the placenta via a specific Fc receptor transcytotic mechanism. Further, it appears that the Fc receptors on the placental syncytiotrophoblast membrane are selective in that immunoglobulins of the IgG1 type have approximately 10-20

fold higher binding affinity for the receptor. In fact, of all the IgG subtypes, IgG1 and 3 have the highest affinity for the receptor, followed by IgG4, and finally IgG2 (30). These results are consistent with those obtained from the cloning of the FcR from a human placenta, which indicate that the receptor is very similar to the FcRII type found on M/M. Although one might argue that transplacental transport of immunoglobulin may be beneficial to the fetus in utero, it could also be argued that specific maternal immunoglobulin raised to a specific pathogen (such as HIV), might facilitate transport across the placenta via an Fc dependent mechanism, to increase infection of the fetus, similar to the mechanism which has evolved to transport IgA across epithelia, via the poly Ig receptor (34). Thus specific CD4-IgG1 fusion proteins, which have been demonstrated to cross the placenta and concentrate in the fetal blood (22), may be detrimental to the fetus, by providing HIV with a novel mechanism to cross the placental barrier.

We have now discovered that a specific CD4-gamma2 chimeric heavy chain homodimer provides advantages relative to those CD4-IgG1 heavy chain homodimers which have been described more than one year ago. Specifically, we have constructed a CD4-gamma2 chimeric heavy chain homodimer which contains the V1V2 domains of CD4 and which is efficiently assembled intracellularly and efficiently secreted from mammalian cells as a homodimer, enabling high recovery and purification from the medium of cells expressing this chimeric heavy chain homodimer. To construct this homodimer, we have used the entire hinge, CH2, and CH3 domains from a human gamma2 heavy chain, which results in a chimeric molecule containing the constant domains of a human IgG2 molecule responsible for dimerization and efficient secretion. This is in contrast to the heavy chain dimers described by Capon and Gregory (20) which include the CH1

domain in the CD4-IgG1 heavy chain dimer, resulting in poor secretion and recovery from cell culture medium of the recombinant molecule. We have also included the entire hinge domain of gamma2 heavy chain in the CD4-gamma2 chimeric heavy chain homodimer of this invention to provide efficient dimerization, since the cysteine residues contained in this domain are responsible for forming the disulphide links to the second chain of the homodimer, positioning the two chains in the correct spatial alignment and facilitating formation of the antigen combining site.

Furthermore, by including the entire hinge domain, we have maintained the segmental flexibility of the heavy chain dimers, thus enabling modulation of biological function such as complement activation and Fc receptor binding (29).

Since IgG2 immunoglobulins have a greatly diminished ability to bind to Fc receptors on monocytes, macrophages, and placental membranes, construction of a CD4-gamma2 chimeric heavy chain homodimer and a CD4-IgG2 chimeric heterotetramer results in chimeric proteins with many advantages that CD4-gamma1 chimeric heavy chain homodimers or CD4-IgG1 chimeric heterotetramers may not possess (20, 23, 24, 26). Furthermore, human IgG2 is significantly less polymorphic than other IgG types and therefore is less likely to be immunogenic when administered to humans. This is in contrast to human IgG1 which contains many allotypes and has a higher probability of being immunogenic when administered to humans.

In addition to the CD4-gamma2 chimeric heavy chain homodimers, we have also constructed CD4-IgG2 heavy chains, which contain the V1V2 domains of CD4 fused to the CH1, hinge, CH2 and CH3 domains of human gamma2 heavy chain. These molecules encode a CD4-IgG2 chimeric heterotetramer, and when co-expressed in the presence of CD4-kappa chimeric

light chains containing the V1 and V2 domains of CD4 fused to the entire constant domain of human kappa light chains (or lambda light chains), enable the production of said heterotetramer. This heterotetramer comprises two CD4-IgG2 chimeric heavy chains and two CD4-kappa chimeric light chains. Producing heavy chains which contain the CH1 domain enables efficient association with the CD4-kappa chimeric light chains, resulting in efficient secretion of a CD4-IgG2 chimeric heterotetramer. These CD4-IgG2 chimeric heterotetramers possess increased serum half-lives and increased avidity for HIV as compared with heavy chain dimers.

Summary of the Invention

This invention provides an expression vector encoding a CD4-gamma2 chimeric heavy chain homodimer. This invention also provides an expression vector encoding the heavy chains of a CD4-IgG2 chimeric heterotetramer. Finally, this invention provides an expression vector encoding the light chains of a CD4-IgG2 chimeric heterotetramer.

Brief Description of the Figures

Figure 1: A) Domain structure of CD4-gamma2 chimeric heavy chain gene; B) Protein structure of CD4-gamma2 chimeric heavy chain homodimer. The sequence shown below is the single letter amino acid code of the junction between CD4 (phe179) and the hinge region of human gamma2 heavy chain. (SEQ ID NO.1) Note that the hinge region of a gamma2 heavy chain contains four cysteines (see text for discussion). Abbreviations: L, leader (signal) sequence of human CD4; V1V2, amino-terminal variable-like domains of human CD4; H, hinge region of human gamma2 heavy chain; CH2 and CH3, second and third constant regions of human gamma2 heavy chain.

Figure 2: A) Domain structure of chimeric genes used to express CD4-IgG2 chimeric heterotetramer. Top, CD4-gamma2 chimeric heavy chain gene; Bottom, CD4-kappa chimeric light chain gene. B) Protein structure of CD4-IgG2 chimeric heterotetramer. Abbreviations: CH1-CH2-CH3, first, second and third constant regions of human gamma2 heavy chain; C-kappa, constant region of human kappa light chain.

Figure 3: DNA and predicted protein sequence of a CD4-gamma2 chimeric heavy chain homodimer (one chain). (SEQ ID NO. 2-3) The numbers at the end of each line indicate the nucleotide positions. The numbers above each line indicate the amino acid positions (given in single letter code). The protein domains are indicated above the sequences by arrows.

Figure 4: DNA and predicted protein sequence of a CD4-IgG2 chimeric heavy chain of the CD4-IgG2 chimeric heterotetramer. (SEQ ID NO. 4-5) The numbers at the end of each line indicate the nucleotide positions. The numbers above each line indicate the amino acid positions (given in single letter code). The protein domains are indicated above the

sequences by arrows.

Figure 5: DNA and predicted protein sequence of a CD4-kappa chimeric light chain of the CD4-IgG2 chimeric heterotetramer. (SEQ ID NO:6-7) The numbers at the end of each line indicate the nucleotide positions. The numbers above each line indicate the amino acid positions (given in single letter code). The protein domains are indicated above the sequences by arrows.

Figure 6: Secretion of CD4-gamma2 chimeric heavy chain homodimer from transfected cells. Cos-M5 cells were mock transfected, transfected with CD4-gamma1 chimeric heavy chain mammalian expression vector DNA, or transfected with CD4-IgG2-pcDNA1. At 48-72 hours post-transfection, the cells were radiolabelled with ^{35}S-methionine. Radiolabelled medium was precipitated with Protein-A sepharose beads. The precipitated proteins were analyzed by SDS-PAGE under reducing or non-reducing conditions and were visualized by fluorography. Lane M, medium from mock transfected cells; Lane 1, medium from cells transfected with CD4-gamma1 chimeric heavy chain mammalian expression vector DNA; Lane 2, medium from cells transfected with CD4-IgG2-pcDNA1 DNA.

Figure 7: Precipitation of HIV-1 gp120 with CD4-gamma2 chimeric heavy chain homodimer. Cos-M5 cells were mock transfected, transfected with CD4-gamma1 chimeric heavy chain mammalian expression vector DNA, or transfected with the CD4-IgG2-pcDNA1. At 48-72 hours post transfection, unlabelled aliquots of medium were incubated with an aliquot of ^{35}S-methionine labelled gp120. The complexes were precipitated with Protein A-sepharose beads. The precipitates were then analyzed by SDS-PAGE followed by fluorography. Lane M, medium from mock transfected cells; Lane 1, medium from cells transfected with CD4-gamma1 chimeric heavy chain mammalian expression vector DNA; Lane

2, medium from cells transfected with CD4-IgG2-pcDNA1 DNA.

Figure 8: Purification of CD4-gamma2 chimeric heavy chain homodimer from CHO cell-conditioned medium. Stable CHO cells constitutively secreting CD4-gamma1 chimeric heavy chain homodimer, or CD4-gamma2 chimeric heavy chain homodimer, were grown in roller bottles. Conditioned medium was passed over a Protein A-sepharose column and bound material was eluted from the column. The peak fractions were identified by SDS-PAGE followed by silver staining and pooled. The purified proteins were then analyzed by SDS-PAGE under reducing conditions followed by silver staining. Lane 1, CD4-gamma1 chimeric heavy chain homodimer; Lane 2, CD4-gamma2 chimeric heavy chain homodimer.

Figure 9: Inhibition of HIV binding to CEM cells by CD4-based molecules. Soluble CD4 (sCD4), partially purified CD4-gamma1, or partially purified CD4-gamma 2 were tested for inhibition of virus binding to CD4-positive cells. Bound virus was detected by indirect immunofluorescence and cytofluorography. Results are expressed as percent inhibition versus concentration of inhibiting agent.

Figure 10: Inhibition of HIV infection of CD4-positive cells by CD4-based molecules. sCD4, partially purified CD4-gamma1, or partially purified CD4-gamma2 were incubated with an HIV-1 inoculum (100 $TCID_{50}$), and mixtures were added to PHA-stimulated lymphocytes and incubated at 37°C overnight. The cells were washed and plated in microculture (1×10^5 cells/culture; 10 cultures per dilution) and monitored for reproductive viral replication by detection of HIV antigen in culture supernates 8 and 12 days later. Results are expressed as percent positive cultures at a given concentration of inhibiting agent.

Figure 11: Purification of CD4-gamma2 chimeric heavy chain

homodimer. Stable CHO cells constitutively secreting CD4-gamma2 chimeric heavy chain homodimer were grown in roller bottles. Conditioned medium was passed over a Protein A-sepharose column and bound material was eluted from the column (see Figure 8). The peak fractions were then pooled and passed over an S-sepharose column. After extensive washes, the CD4-gamma2 chimeric heavy chain homodimer was eluted with 50mM BES pH 7.0, 500mM NaCl. The peak fractions were identified by SDS-PAGE followed by silver staining, pooled, and concentrated. The pooled, concentrated CD4-gamma2 chimeric heavy chain homodimer was then applied to a Sephacryl S-300HR column preequilibrated and run with PBS. The peak fraction corresponding to purified CD4-gamma2 chimeric heavy chain homodimer was identified by SDS-PAGE followed by silver staining. The peak fractions were then pooled and concentrated. The purified protein was then analyzed by SDS-PAGE under non-reducing and reducing conditions followed by silver staining. Lane 1: approximately 1.5 μg protein run under non-reducing conditions, Lane 2: approximately 1.5μg protein run under reducing conditions.

Figure 12: Secretion of CD4-IgG2 chimeric heterotetramer from stably transfected cells. CHO cells stably expressing both CD4-IgG2 chimeric heavy chains and CD4-kappa chimeric light chains were radiolabelled with ^{35}S-methionine and cysteine. Radiolabelled medium was precipitated with Protein-A sepharose beads. (A) The precipitated proteins were analyzed by SDS-PAGE under non-reducing conditions, and were visualized by fluorography. Lane 1: medium from untransfected CHO cells, Lane 2: medium from cells stably expressing both the CD4-IgG2 chimeric heavy chains, and CD4-kappa chimeric light chains. (B) An identical sample to that run in lane 2 from (A) was run on SDS-PAGE under non-reducing conditions. The lane from this SDS-PAGE gel was excised and the proteins reduced by incubation of the gel

slice for 45 minutes at 4°C in equilibration buffer (62.5mM TrisHCl pH 6.8, 2.3% SDS 5% ß-mercaptoethanol, 10% glycerol). After incubation of the gel slice under reducing conditions, the proteins contained within the gel were analyzed by SDS-PAGE and visualized by fluorography.

Detailed Description of the Invention

Five expression vectors and two plasmids designated CD4-IgG2-Rf, CD4-IgG1-Rf, CD4-IgG1HC-pRcCMV, CD4-IgG2HC-pRcCMV, CD4-kLC-pRcCMV, CD4-IgG1-pcDNA1, and CD4-IgG2-pcDNA, respectively have been deposited with the American Type Culture Collection, Rockville, Maryland, U.S.A. 20852, under ATCC Accession Nos. 40949, 40950, 75192, 75193, 75194, 40951, and 40952, respectively. These deposits were made pursuant to the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (Budapest Treaty).

Specifically, the invention provides an expression vector designated CD4-IgG2-pcDNA1 (ATCC No. ~~40951~~ 40952) encoding a CD4-gamma2 chimeric heavy chain homodimer. The invention additionally provides a CD4-gamma2 chimeric heavy chain homodimer encoded by this expression vector or any other expression vector having the same DNA coding region inserted therein. Specifically, the invention also provides expression vectors designated CD4-IgG2HC-pRcCMV (ATCC No. 75193), and CD4-kLC-pRcCMV (ATCC No. 75194), encoding a CD4-IgG2 chimeric heavy chain and a CD4-kappa chimeric light chain. The invention additionally provides a CD4-IgG2 chimeric heterotetramer encoded by these expression vectors or any other expression vector having the same DNA encoding region inserted therein.

In accordance with the invention, numerous vector systems for expression may be employed. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MOMLV) or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be

selected by introducing one or more markers which allow selection of transfected host cells. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, e.g., antibiotics, or resistence to heavy metals such as copper or the like. The selectable marker gene can be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcriptional promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama. (37)

Thus, the invention further provides a method of producing a CD4-gamma2 chimeric heavy chain homodimer. This method comprises

a) transfecting a mammalian cell with an expression vector for producing the CD4-gamma2 chimeric heavy chain homodimer;
b) culturing the resulting transfected mammalian cell under conditions such that CD4-gamma2 chimeric heavy chain homodimer is produced; and
c) recovering the CD4-gamma2 chimeric heavy chain homodimer so produced.

Once the vector or DNA sequence containing the constructs has been prepared for expression, the expression vectors may be transfected or introduced into an appropriate mammalian cell host. Various techniques may be employed such as protoplast fusion, calcium phosphate precipitation, electroporation or other conventional techniques. In the case of protoplast fusion, the cells are grown in media and screened for the appropriate activity. Expression of the gene(s) results in production of the fusion protein which corresponds to one chain of the CD4-gamma2 chimeric heavy

chain homodimer. This fusion protein may then be treated to form the chimeric heavy chain homodimer.

Further, methods and conditions for culturing the resulting transfected cells and for recovering the chimeric heavy chain homodimer so produced are well known to those skilled in the art and may be varied or optimized depending upon the specific expression vector and mammalian host cell employed.

In accordance with the claimed invention, the preferred host cells for expressing the chimeric heavy chain homodimers of this invention are mammalian cell lines, including, for example, monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line 293; baby hamster kidney cells (BHK); Chinese hamster ovary-cells-DHFR (CHO); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); mouse cell line (C127) and myeloma cell lines.

The invention further provides a method of inhibiting the HIV infection of a CD4+ cell which comprises treating the CD4+ cell with the CD4-gamma2 chimeric heavy chain homodimer in an amount which is effective to inhibit infection of the cell.

Additionally, the invention provides a method of preventing a subject from being infected with HIV which comprises administering to the subject the CD4-gamma2 chimeric heavy chain homodimer in an amount which is effective to prevent the subject from being infected with HIV.

Although the invention encompasses the administration of the chimeric heavy chain homodimer to various subjects, AIDS patients are of particular interest. Further, methods of

administering the homodimer are well known in the art and include, merely by way of example, subcutaneous, intramuscular and intravascular injection, alone or in combination with other agents such as AZT or DDI.

Further provided is a method of treating a subject infected with HIV so as to block the spread of HIV infection which comprises administering to the subject an amount of the CD4-gamma2 chimeric heavy chain homodimer in an amount which is effective to block the spread of HIV infection.

For example, the homodimer may be administered to patients having HIV infection at a dosage capable of maintaining a concentration of greater than about 100 ng of CD4-gamma2 chimeric heavy chain homodimer/ml plasma. For CD4-gamma2 chimeric heavy chain homodimer variants having different molecular weights, about 2 picomoles of soluble receptor per ml of plasma, an amount for example, sufficient to establish a stoichiometric equivalence with native (membrane bound) and soluble receptor is administered. Typically, the dosage of soluble CD4 is about 100 μg/kg of patient weight/day.

The foregoing method may be used to help prevent the spread of the HIV virus within the body of a HIV infected patient. Additionally, CD4-gamma2 chimeric heavy chain homodimer may be administered as a prophylactic measure to render a subject's blood less susceptible to the spread of the HIV virus. Such prophylactic administration includes administration both prior to HIV contact or shortly thereafter, or both.

A pharmaceutical composition which comprises the CD4-gamma2 chimeric heavy chain homodimer of thus invention in an amount effective to inhibit HIV infection of a CD4+ cell and a pharmaceutically acceptable carrier is further provided.

Pharmaceutically acceptable carriers are well known in the art to which the present invention pertains and include, but are not limited to, 0.01-0.1M and preferably 0.05 M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like. (38)

The invention further provides a composition of matter comprising a CD4-gamma2 chimeric heavy chain homodimer and a toxin linked thereto.

Some example of toxins are the deglycosylated A chain of ricin, domains II or III of Pseudomonas exotoxin A, Diphtheria toxin, or a non-peptidyl cytotoxin. These toxins may be linked using conventional in vitro protein cross-linking agents (39-41). Additionally the toxins may be linked by recombinant synthesis as a fusion protein (see for example U.S. Patent 4,765,382).

The invention also provides a diagnostic reagent comprising a CD4-IgG2 chimeric heavy chain homodimer and a detectable marker linked thereto. By employing a molecule which binds to the HIV virus and additionally has attached to it a

detectable marker, one may identify cells which are infected with HIV. Examples of conventional detectable markers includes radioisotopes such as I125, chromophores, and fluorophores.

Thus, the chimeric heavy chain homodimer of the invention may be used in an assay for HIV or SIV viral infection in a biological sample by contacting a sample derived from an animal suspected of having an HIV or SIV infection, with the homodimer of the invention, and detecting whether a complex forms with gp120, either alone or on the surface of an HIV-infected cell. For this purpose the homodimer may be labeled with a detectable marker or may be unlabeled and then be detected with another reagent which is detectably labeled and is specifically directed to the homodimer or to a complex between it and gp120.

For example, a biological sample may be treated with nitrocellulose, or another solid support which is capable of immobilizing cells, cell particles or soluble protein. The support may then be washed with suitable buffers followed by treatment with the chimeric heavy chain homodimer which may be detectably labeled. The solid phase support may then be washed with buffer a second time to remove unbound fusion protein and the labeled homodimer detected.

In carrying out the assay the following steps may be employed.

a) contacting a sample suspected of containing gp120 with a solid support to effect immobilization of gp120, or cells which express gp120 on their surface;

b) contacting said solid support with the detectably labeled chimeric heavy chain homodimer of the invention;

c) incubating said detectably labeled homodimer with said support for a sufficient amount of time to allow the homodimer to bind to the immobilized gp120 or cell which expresses gp120 on its surface;

d) separating the solid phase support from the incubation mixture obtained in step c); and

e) detecting bound labeled homodimer and thereby detecting gp120.

Such a method may be formatted either as a qualitative or as a quantitative test using methods well known in the art.

Alternatively, labeled homodimer-gp120 complex may be separated from a reaction mixture by contacting the complex with an immobilized antibody or protein which is specific for an immunoglobulin or, e.g., protein A, protein G, or anti-IgG antibodies. Such anti-immunoglobulin antibodies may be monoclonal or polyclonal. The solid support may then be washed with suitable buffers to obtain an immobilized gp120-labeled homodimer-antibody complex. The label on the homodimer may then be detected so as to measure endogenous gp120, and thereby detect the presence of HIV.

In one embodiment of the invention, a method for detecting HIV or SIV viral infection in a sample is provided comprising:

a) contacting a sample suspected of containing gp120 with a CD4-gamma2 chimeric heavy chain homodimer in accordance with this invention, and the Fc portion of an immunoglobulin chain; and

b) detecting whether a complex is formed.

The invention also provides a method of detecting gp120 in a sample comprising:

a) contacting a mixture obtained by contacting a sample suspected of containing gp120 with a homodimer of this invention, and the Fc portion of an immunoglobulin chain, with an Fc binding molecule, such as an antibody, protein A, or protein G, which is immobilized on a solid phase support and is specific for the homodimer, to obtain a gp120-homodimer immobilized antibody complex,

b) washing the solid phase support obtained in step (a) to remove unbound homodimer; and

c) detecting the homodimer.

Of course, the specific concentrations of unlabeled or detectably labeled homodimer and gp120, the temperature and time of incubation, as well as other assay conditions, may be varied depending on various factors including the concentration of gp120 in the sample, the nature of the sample, and the like. Those skilled in the art are readily able to determine operative and optimal assay conditions for each determination.

Also provided is an enzyme-linked immunoadsorbent assay (ELISA) to detect and quantify soluble CD4 (sCD4) or CD4 chimeric proteins. In carrying out the assay, the process comprises:

a) contacting a sample containing sCD4 with a solid support to immobilize soluble sCD4;

b) contacting said solid support with the detectably labeled monoclonal antibody OKT4a alone, or with a sample containing sCD4 or CD4 chimeric proteins and OKT4a;

c) incubating said detectably labeled OKT4a containing media for sufficient time to allow for binding to immobilized SCD4;

d) separating the solid phase support from the

incubation mixture in step (c);

e) detecting the bound OKT4a and thereby quantifying the amount of CD4 contained in the sample.

The invention further provides an expression vector encoding the heavy chains of a CD4-IgG2 chimeric heterotetramer, designated CD4-IgG2HC-pRcCMV (ATTC No. 75193). The invention also provides a CD4-IgG2 chimeric heterotetramer, the heavy chains of which are encoded by this expression vector or another vector containing the same coding sequence.

Additionally, the invention provides an expression vector encoding the light chains of a CD4-IgG2 chimeric heterotetramer, designated CD4-kLC-pRcCMV (ATCC No. 75194). Finally, the invention provides a CD4-IgG2 chimeric heterotetramer, the light chains of which are encoded by the CD4-kLC-pRcCMV expression vector or another vector containing the same coding sequence.

Further, the invention provides a CD4-IgG2 chimeric heterotetramer both the heavy and light chains of which are encoded by the aforementioned expression vectors.

The invention further provides a method of producing such a CD4-IgG2 chimeric heterotetramer. This method comprises:

a) cotransfecting a mammalian cell with the expression vector for producing the light chains of a CD4-IgG2 chimeric heterotetramer and an expression vector encoding a light chain;

b) culturing the resulting cotransfected mammalian cell under conditions such that CD4-IgG2 chimeric heterotetramer is produced; and

c) recovering the CD4-IgG2 chimeric heterotetramer so produced.

Methods of cotransfecting mammalian cells are well known in the art and include those discussed hereinabove. Similarly, expression vectors encoding light chains are well known in the art.

The invention additionally provides a method of producing a CD4-IgG2 chimeric heterotetramer which comprises:

a) cotransfecting a mammalian cell with the expression vector for producing the light chains of a CD4-IgG2 chimeric heterotetramer and with an expression vector encoding an IgG1 heavy chain;

b) culturing the resulting cotransfected mammalian cell under conditions such that a CD4-IgG2 chimeric hetero-tetramer is produced; and

c) recovering the CD4-IgG2 chimeric heterotetramer so produced.

Further the invention provides a method of producing an CD4-IgG2 chimeric heterotetramer which comprises:

a) cotransfecting a mammalian cell with the expression vector for producing the heavy chains of a CD4-IgG2 chimeric heterotetramer and an expression vector for producing the light chains of an CD4-IgG2 chimeric heterotetramer;

b) culturing the resulting cotransfected mammalian cell under conditions such that the CD4-IgG2 chimeric heterotetramer is produced; and

c) recovering the CD4-IgG2 chimeric heterotetramer so produced.

The invention also includes a method of inhibiting HIV infection of a CD4+ cell which comprises treating the CD4+ cell with either a CD4-IgG2 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG2HC-pRcCMV; a CD4-IgG2 chimeric heterotetramer, the light chains of which are encoded by the

expression vector designated CD4-kLC-pRcCMV; or a CD4-IgG2 chimeric heterotetramer, both the heavy and the light chains of which are encoded by both of the above expression vectors, in an amount effective to inhibit infection of the cell.

The invention further provides a method of preventing a subject from being infected with HIV. This method comprises administering to the subject either a CD4-IgG2 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG2HC-pRcCMV; a CD4-IgG2 chimeric heterotetramer, the light chains of which are encoded by the expression vector designated CD4-kLC-pRcCMV; or a CD4-IgG2 chimeric heterotetramer, both the heavy and the light chains of which are encoded by the above expression vectors, in an amount which is effective to prevent the subject from being infected with HIV.

The invention also provides a method of treating a subject infected with HIV so as to block the spread of HIV infection. This method comprises administering to the subject either a CD4-IgG2 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG2HC-pRcCMV; a CD4-IgG2 chimeric heterotetramer, the light chains of which are encoded by the expression vector designated CD4-kLC-pRcCMV; or a CD4-IgG2 chimeric heterotetramer, both the heavy and the light chains of which are encoded by the above-described expression vectors, in an amount effective to block spread of HIV infection, for example, within the subject or an AIDS patients body.

The invention also provides a pharmaceutical composition which comprises either a CD4-IgG2 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG2HC-pRcCMV; a CD4-IgG2 chimeric

heterotetramer, the light chains of which are encoded by the expression vector designated CD4-kLC-pRcCMV, or a CD4-IgG2 chimeric heterotetramer, both the heavy and the light chains of which are encoded by the above-described expression vectors, in an amount effective to inhibit HIV infection of a CD4+ cell, and a pharmaceutically acceptable carrier.

Further provided by the invention is a composition of matter comprising either a CD4-IgG2 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG2HC-pRcCMV; a CD4-IgG2 chimeric heterotetramer, the light chains of which are encoded by the expression vector designated CD4-kLC-pRcCMV, or a CD4-IgG2 chimeric heterotetramer, both the heavy and the light chains of which are encoded by the above-described expression vectors, and a toxin linked thereto.

In one embodiment of the invention, the toxin is the deglycosylated A chain of ricin, domains II or III of Pseudomonas exotoxin A, Diphtheria toxin, or a non-peptidyl cytotoxin.

The invention further provides a diagnostic reagent either comprising a CD4-IgG2 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG2HC-pRcCMV; a CD4-IgG2 chimeric heterotetramer the light chains of which are encoded by the expression vector designated CD4-kLC-pRcCMV; or a CD4-IgG2 chimeric heterotetramer both the heavy and the light chains of which are encoded by both of those expression vectors, and a detectable marker linked thereto. Examples of suitable detectable markers are radioisotopes, chromophores or fluorophores.

In order to facilitate understanding of the following examples, certain frequently occurring methods and/or terms

are best described in Maniatis et al. (42)

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Experimental Details

A. **Materials and Methods**

1. Construction of CD4-gamma2 chimeric heavy chain gene encoding CD4-gamma2 chimeric heavy chain homodimer:

The human CD4 cDNA was excised from the plasmid pSP6T4 (4) as an EcoR1/Stu1 restriction fragment. The 0.70 kilobase fragment was isolated and cloned into EcoR1/Sma1 digested M13mp18. This intermediate vector (M13mp18(CD4)) was then isolated, linearized with Pst1, purified, and treated with Bacterial Alkaline Phosphatase (BAP). The 2.0 Kb Pst1/Pst1 fragment from the plasmid pBr gamma2 containing the human gamma2 heavy chain gene (36) (containing the hinge, CH2, and CH3 exons) was isolated and cloned into the BAP-treated M13mp18/CD4 vector. Resulting recombinants were then screened for the correct orientation of the Pst1 fragment (with respect to the CD4 sequence) to obtain a vector which contains in tandem CD4(EcoR1/Stu1) - gamma2(Pst1/Pst1). To obtain a CD4-gamma2 chimeric heavy chain gene, oligonucleotide-mediated site-directed mutagenesis was performed to juxtapose the CD4 and gamma2 heavy chain DNA sequences, ligating the CD4 sequence in frame to the hinge exon. The resulting chimeric DNA molecule encodes a protein containing the V1V2 domains of CD4 followed by the hinge, CH2, and CH3 domains of gamma2 heavy chain (Figure 1A). Mutagenesis was performed on single-stranded DNA isolated from recombinant phage from transformed TG1 cells (Amersham). Briefly, template DNA was annealed with a 34-mer oligonucleotide (5'-GACACAACATTTGCGCTCGAAAGCTAGCACCACG-3', SEQ ID NO. 8), containing sequences which join the last codon encoding Phe(179) from V1V2 of CD4 to the first codon of the hinge for IgG2 (encoding Glu) (Figures 1A and 3). After second strand synthesis, double stranded DNA was transformed into

competent TG1 cells. Isolated plaques were then grown in fresh TG1 cells and single stranded DNA was purified for DNA sequencing. All mutations were verified and confirmed by dideoxy sequencing using the Sequenase system (USB). Plaques containing the chimeric gene with the correct sequence were then grown in TG1 cells, and Rf DNA (designated CD4-IgG2-Rf) was isolated from the cells.

2. Construction of Mammalian Expression Vector Encoding CD4-gamma2 chimeric heavy chain homodimer:

The CD4-gamma2 chimeric heavy chain gene was isolated from the recombinant Rf DNA following Rf linearization with EcoR1. The EcoR1 sites in the linearized DNA were filled in with the Klenow fragment of DNA polymerase I. The flush ended DNA was then ligated overnight at 15 degrees Celsius with T4 DNA ligase to a 100-fold molar excess of HindIII linkers. After heat inactivation of T4 DNA ligase for 15 minutes at 70 degrees Celsius, the HindIII-linkered DNA was extensively digested with HindIII to liberate a fragment containing the CD4-gamma2 chimeric heavy chain gene. This HindIII fragment was then purified and ligated to the expression vector pcDNA-1 (Invitrogen), which was previously digested with HindIII and BAP treated. The resulting plasmid was then transformed into MC1061/P3 cells. Plasmid DNA was isolated from recombinant clones, and verification of the presence of the HindIII insert and orientation of the insert with respect to the cytomegalovirus (CMV) promoter in the plasmid was made by restriction enzyme analysis. The resulting mammalian expression plasmid which encodes a CD4-gamma2 chimeric heavy chain homodimer is designated CD4IgG2-pcDNA1.

3. Expression of CD4-IgG2-pcDNA1 in mammalian cells:

a. Transient expression.

CosM5 cells grown in DMEM containing 10% fetal calf serum were split to 75% confluence. On the following day, the cells were transfected for 16-20 hours with 10 micrograms of CsCl-purified plasmid CD4IgG2-pcDNA1 DNA by the standard CaPO(4) precipitation technique. After transfection, fresh medium was added to the cells. Analysis of the products synthesized 48-72 hours post-transfection was performed by radiolabelling of transfectants with ^{35}S-methionine for 12-18 hours followed by precipitation of media and cell lysates using anti-CD4 antibodies or by incubation with Protein A-sepharose beads alone followed by SDS-PAGE under reducing or non-reducing conditions (Figure 6). In addition, analysis of media and cell lysates was performed 48-72 hours post-transfection by standard Western blotting procedures.

b. Stable expression.

Dhfr-Chinese hamster ovary cells (CHO) were transfected with 20 micrograms of CsCl purified DNA in a 1000:1 molar ratio of CD4IgG2-pcDNA1:p410 (p410 is an expression plasmid containing the dhfr gene), although other ratios may also be used. Approximately 3-5 days post-transfection, cells were placed in selective medium (nucleoside-free alpha MEM containing 10% dialyzed fetal calf serum). Approximately 10-15 days post-selection, individual cell clones were picked and analyzed for stable expression of CD4-gamma2 chimeric heavy chain homodimer by several screening techniques, such as ELISA and precipitation with Protein A-sepharose beads followed by SDS-PAGE under reducing and non-reducing conditions. Clones expressing the highest levels were subjected to successive rounds of amplification of the newly introduced DNA sequences in increasing concentrations of methotrexate. Stable CHO cell lines were thus generated which secrete between 10-100 micrograms/milliliter of CD4-

gamma2 chimeric heavy chain homodimer.

4. Purification of CD4-gamma2 chimeric heavy chain homodimer from CHO conditioned media:

CD4-gamma2 chimeric heavy chain homodimer was purified in a single step using Protein A-Sepharose column chromatography. CHO cells secreting CD4-gamma2 chimeric heavy chain homodimer were grown to high density in roller bottles in medium containing alpha MEM with 10% IgG-free fetal calf serum. Conditioned media was collected, clarified by centrifugation, and diluted 1:1 with PBS with/or without detergent (i.e. Tween) in this and subsequent buffers. The diluted media was then applied to a 5ml column of Protein A-Sepharose fast flow previously equilibrated with PBS, at a flow rate of 60ml/hour. After extensive washing, the specifically bound material was eluted with 100mM glycine/HCl, pH 3.5, directly into an aliquot of 1M Tris.HCl pH 8.0 to immediately neutralize the eluted fractions. The fractions were then analyzed by SDS-PAGE under reducing and non-reducing conditions followed by silver staining and pooled (Figure 8).

The pooled fractions were then applied to a 10 ml column of S-sepharose fast flow previously equilibrated with 50mM BES pH 7.0 at a flow rate of 120ml/hr. After application of sample, a step elution gradient (consisting of the following 4 steps: 5 column volumes of 50mM BES pH 7.0, 4 column volumes of 50mM BES pH 7.0, 100mM NaCl, 6 column volumes of 50mM BES pH 7.0 225mM NaCl, followed by 8 column volumes of 50mM BES pH 7.0, 500mM NaCl) was employed for specific elution of the CD4-gamma2 chimeric heavy chain homodimer. The CD4-gamma2 chimeric heavy chain homodimer was eluted from the column in 50mM BES pH 7.0, 500mM NaCl. The peak fractions were then pooled and concentrated to yeild a final protein concentration of at least 1 mg/ml. The pooled and

concentrated fractions were then applied to a 120 ml column of Sephacryl S-300HR previously equilibrated with PBS, at a flow rate of 8ml/hr. The CD4-gamma2 chimeric heavy chain homodimer fraction was specifically eluted in PBS, and concentrated to at least 1mg/ml.

5. Demonstration of binding of CD4-gamma2 chimeric heavy chain homodimer to the HIV envelope glycoprotein gp120:

CosM5 transfectants expressing CD4-gamma2 chimeric heavy chain homodimer were incubated for 72 hours in DMEM containing 10% IgG-free fetal calf serum. Unlabelled medium was then collected and used to precipitate ^{35}S-methionine-radiolabelled HIV gp120. After incubation of CD4-gamma2 chimeric heavy chain homodimer containing medium containing ^{35}S-methionine-labelled gp120, the complexes were adsorbed to Protein A-sepharose. Protein A-sepharose complexes were recovered by centrifugation, and the precipitates were analyzed by SDS-PAGE under reducing conditions followed by fluorography (Figure 7). Alternatively, aliquots of purified CD4-gamma2 chimeric heavy chain homodimer from CHO cells were also used to precipitate ^{35}S-radiolabelled gp120 using the same procedure.

6. Determination of plasma half-life and placental transfer of CD4-gamma2 chimeric heavy chain homodimer:

Determination of the plasma half-life and placental transfer are performed by well established techniques. Briefly, rabbits or monkeys are injected intravenously or intramuscularly with purified CD4-gamma2 chimeric heavy chain homodimer. At various time points post-injection, plasma samples are taken, and the quantity of the CD4-gamma2 chimeric heavy chain homodimer present in the serum is measured by ELISA. In addition, pregnant monkeys are also injected either IV or IM with CD4-gamma2 chimeric heavy

chain homodimer and the concentration determined in the cord blood and the serum of the newborn monkey. Determination and comparison of the quantity of the CD4-gamma2 chimeric heavy chain homodimer in the mother's serum as well as in the cord blood and serum of the newborn indicates the relative rate of transport across the placenta of these molecules.

7. Determination of FcR binding and macrophage infectivity of CD4-gamma2 chimeric heavy chain homodimer.

Determination of FcR binding and macrophage infectivity of CD4-gamma2 chimeric heavy chain homodimer are performed by well established techniques. For these studies, U937 cells (a human monocytic cell line expressing FcR1 and FcRII), purified monocyte/macrophage populations from human peripheral blood, and Hela cells constitutively expressing recombinant human FcRs are utilized. In addition, monoclonal antibodies specific for FcR1 and FcRII are commercially available. Briefly, radiolabelled monomeric or aggregated CD4-gamma2 chimeric heavy chain homodimer is incubated with the above cells and appropriate control cells at 4 degrees Celsius over various time points. At the end of each incubation, the cells are solubized and the cell-associated radioactivity is determined to establish the amount of CD4-gamma2 chimeric heavy chain homodimer specifically bound to each cell type. As controls, radiolabelled normal monomeric or aggregated human IgG2 are used to determine the levels of specific antibody binding. Furthermore, competing the radiolabelled component with unlabelled monomeric or aggregated normal human IgG2, or monoclonal antibodies to FcRI or FcRII, will establish the binding efficiency and specificity of CD4-gamma2 chimeric heavy chain homodimer to each cell type.

To ascertain whether the CD4-gamma2 chimeric heavy chain

homodimer mediates enhancement of HIV infection of monocytes/macrophages, HIV-1 is incubated with media alone or either monomeric or aggregated CD4-gamma2 chimeric heavy chain homodimer at several dilutions. As controls, sera from normal individuals and HIV-infected individuals are used (31). After incubation for one hour at 4 degrees Celsius, the 'opsonized' virus is added to the cell types described in the paragraph above. At various time points after infection, the media is harvested and assayed for viral reverse transcriptase activity to determine the degree of viral infection. As controls, sCD4, OKT4a or Leu3a are included during the infection of the cells. In addition, various dilutions of the CD4-gamma2 chimeric heavy chain homodimer and appropriate controls are first incubated with the cells at 4 degress Celsius to allow binding. HIV is then added and infection assayed by viral reverse transcriptase activity.

8. <u>HIV binding assay</u>:

Binding of HIV was performed as previously described (43, 44). Briefly, concentrated HIV-1 preparations were incubated with various dilutions of sCD4, CD4-gamma2, or CD4-gamma2, for 30 minutes and then added to 5 x 10^5 CEM cells. Bound virus was detected by indirect immunofluorescence and cytofluorography as previously described (44).

9. <u>Neutralization assay</u>:

The microculture assay for productive viral replication was as previously described (43, 45). Briefly, dilutions of sCD4, CD4-gamma2, or CD4-gamma2 were incubated for 30 minutes with 100 $TCID_{50}$ HIV-1 at room temperature. The mixtures were added to PHA-stimulated lymphocytes and incubated at 37°C overnight. The cells were then washed and

plated in microculture at 1 x 10^5 cells/culture; and 10 cultures per dilution were monitored for reproductive viral replication by detection of HIV antigen in culture supernates 8 and 12 days later.

B. Construction of CD4-IgG2 chimeric heavy chain and CD4-kappa chimeric light chain for expression of CD4-IgG2 chimeric heterotetramer:

1. Introduction

This invention describes a CD4-gamma2 chimeric heavy gene encoding a CD4-gamma2 chimeric heavy chain homodimer which is efficiently secreted from transformed mammalian cells. This chimeric molecule was designed to contain sequences from the human IgG2 heavy chain which allow for efficient homodimer assembly and secretion. The CH1 region of the IgG heavy chains is responsible for retaining heavy chain molecules intracellularly and for formation of heterotetramers with light chains (25). In order to efficiently produce CD4-gamma2 chimeric heavy chain homodimers, the CD4-gamma2 chimeric heavy chain gene described above specifically lacks the CH1 domain. The resulting homodimer contains two CD4 V1V2 moieties and therefore has the potential of being bivalent with respect to gp120 binding and having enhanced avidity for HIV compared to sCD4.

In addition, this invention describes the construction of CD4-IgG2 chimeric heterotetramers which contain two heavy chains and two light chains. The resulting heterotetramer, containing two or four CD4 V1V2 moieties, and has the potential of being tetravalent with respect to gp120 binding and having enhanced avidity for HIV compared to sCD4. The CD4-IgG2 chimeric heavy chain gene used to produce CD4-IgG2 chimeric heterotetramer contains the entire heavy chain

constant region, including the CH1 domain. The inclusion of the CH1 domain facilitates efficient intracellular association with light chains, affording the potential for secreted, disulfide-bonded heterotetramers. Both the CD4-IgG2 chimeric heavy chain gene and the CD4-kappa chimeric light chain gene contain the V1V2 domains of CD4. Efforts to express CD4-IgG2 chimeric heavy chains or CD4-kappa chimeric light chains (either alone or in combination) containing only the V1 domain of CD4 were unsuccessful.

2. Construction of CD4-IgG2 chimeric heavy chain expression vector and CD4-kappa chimeric light chain expression vector for production of CD4-IgG2 chimeric heterotetramers.

a. Construction of CD4-IgG2 chimeric heavy chain mammalian expression vector.

The human CD4 cDNA sequence is excised from the plasmid pSP6T4 (4) as an EcoR1/Stu1 restriction fragment. The 0.70 kilobase fragment is isolated and cloned into EcoR1/Sma1-digested M13mp18. The resulting vector (M13mp18(CD4)) is then isolated and digested with BamH1. The BamH1 sites of the M13mp18(CD4) are made flush ended with the Klenow fragment of DNA polymerase 1. After heat inactivation of the polymerase for 15 minutes at 65 degrees Celsius, the linearized M13mp18(CD4) vector is then digested with Pst1 and purified.

In order to excise a fragment containing the CH1 exon of the human gamma2 heavy chain gene, the plasmid pBr gamma2 (36) is digested with SacII, and the SacII sites are then made flush using T4 DNA polymerase. After heat inactivation of the polymerase, the fragment is then digested with Pst1. The resulting SacII(flush)-Pst1 fragment containing the CH1 exon is then purified and ligated to the M13mp18(CD4) vector

described in the above paragraph. After transformation of competent TG1 cells, the resulting recombinants are screened by restriction analysis for the presence of both CD4 and CH1 sequences which contain in tandem CD4 (EcoR1/Stu1) - CH1 (SacII(flush)/Pst1). Oligonucleotide-mediated site-directed mutagenesis is then performed to juxtapose the CD4 and CH1 sequences in frame. The resulting chimeric DNA molecule contains the V1V2 domains of CD4 fused to the CH1 domain of gamma2 heavy chain. Mutagenesis is performed on single-stranded DNA isolated from recombinant phage from transformed TG1 cells (Amersham). Template DNA is annealed with a 33-mer oligonucleotide (5'-GGGCCCTTGGTGGA GGCGAAAGCTAGCACCACG-3') containing sequences which join the last codon encoding Phe (179) from V1V2 of CD4 to the first codon of the CH1 domain for gamma2 heavy chain (encoding Ala). After second strand synthesis, double stranded DNA is transformed into competent TG1 cells. Isolated plaques are then grown in fresh TG1 cells and single-stranded DNA is purified for DNA sequencing. All mutations are confirmed by dideoxy sequencing using the Sequenase system (USB). Plaques containing the chimeric genes with the correct sequence as determined by restriction analysis are then grown in TG1 cells, and the Rf DNA is isolated from the cells.

Rf DNA from the CD4-CH1 chimeric gene is then linearized by digestion with Pst1. The Pst1 linearized vector is then BAP treated and ligated to the Pst1-Pst1 DNA fragment of the plasmid pBr gamma2 containing the hinge, CH2, and CH3 exons of the human gamma2 heavy chain gene. The correct orientation of the Pst1-Pst1 fragment with respect to the chimeric CD4-CH1 fragment is then verified by restriction analysis. The resulting chimeric gene encodes a protein containing the V1V2 domains of CD4 followed by the CH1, hinge, CH2, and CH3 regions of gamma2 heavy chain (Figures 2A, 2B, and 4).

The CD4-IgG2 chimeric heavy chain DNA molecule is isolated from the recombinant Rf DNA following Rf linearization with EcoR1. The EcoR1 sites in the linearized DNA are filled in with the Klenow fragment of DNA polymerase I. The flush ended DNA is then ligated overnight at 15 degrees Celsius with T4 DNA ligase to a 100-fold molar excess of HindIII linkers. After heat inactivation of T4 DNA ligase for 15 minutes at 70 degrees Celsius, the HindIII-linkered DNA is extensively digested with HindIII to liberate a fragment containing the CD4-IgG2 chimeric heavy chain gene. This HindIII fragment is then purified and ligated to the expression vector pcDNA-1 (Invitrogen), which was previously digested with HindIII and BAP treated. The resulting plasmid is then transformed into MC1061/P3 cells. Plasmid DNA is isolated from recombinant clones, and verification of the presence of the HindIII insert and orientation of the insert with respect to the cytomegalovirus (CMV) promoter in the plasmid is made by restriction analysis. The resulting mammalian expression plasmid which encodes a CD4-IgG2 chimeric heavy chain is designated CD4-IgG2HC-pRcCMV.

b. <u>Construction of a CD4-kappa chimeric light chain mammalian expression vector</u>:

The human kappa light chain constant region is excised from the plasmid pCNkappa light as an Mse1 fragment. The purified Mse1 fragment is then made flush ended using the Klenow fragment of DNA polymerase 1. M13mp18 Rf is then linearized with HincII, and the flush ended Mse1 kappa light chain fragment is ligated to M13mp18 at the flush ended HincII site in the vector. After transformation of TG1 cells, the recombinants are confirmed for the presence of the insert and the correct orientation within the vector by restriction analysis. Rf is purified from infected TG1

cells and digested with EcoR1 and Sma1. The purified vector containing the kappa light chain constant region is then ligated to the EcoR1/Stu1 fragment of the human CD4 cDNA described above. The resulting recombinants are then verified for the presence and orientation of both inserts containing in tandem CD4 (EcoR1/Stu1) - Ckappa (MseI(flush)/MseI(flush)), and single-stranded DNA is purified for oligonucleotide-mediated site directed mutagenesis. Template DNA is annealed to a 33-mer oligonucleotide (5'-GATGGTGCAGCCACAGTGAAAGCTAGCACCACG-3') (SEQ ID NO.10) containing sequences which join the last codon encoding Phe(179) from V1V2 of CD4 to the first codon of the kappa light chain constant domain (encoding thr). After second strand synthesis, double-stranded DNA is transformed into competent TG1 cells, and isolated plaques are grown in fresh TG1 cells for DNA sequencing. The presence of the mutation is confirmed by dideoxy sequencing. Plaques containing chimeric genes with the correct sequence are then grown in TG1 cells, and Rf DNA is isolated from the cells. The resulting DNA molecule encodes a protein containing the V1V2 domains of CD4 followed by the constant region of kappa light chains (Figures 2A, 2B and 5).

The CD4-kappa chimeric light chain DNA molecule is isolated from the recombinant Rf DNA following Rf linearization with EcoR1. The EcoR1 sites in the linearized DNA are filled in with the Klenow fragment of DNA polymerase I. The flush ended DNA is then ligated overnight at 15 degrees Celsius with T4 DNA ligase to a 100-fold molar excess of HindIII linkers. After heat inactivation of T4 DNA ligase for 15 minutes at 70 degrees Celsius, the HindIII linkered DNA is extensively digested with HindIII to liberate a fragment containing the CD4-kappa chimeric light chain gene. This HindIII fragment is then purified and ligated to the expression vector pcDNA-1 (Invitrogen), which was previously digested with HindIII and BAP treated. The resulting

plasmid is then transformed into MC1061/P3 cells. Plasmid DNA is isolated from recombinant clones, and verification of the presence of the HindIII insert and orientation of the insert with respect to the cytomegalovirus (CMV) promoter in the plasmid is made by restriction enzyme analysis. The resulting mammalian expression plasmid which encodes a CD4-kappa chimeric light chain is designated CD4-kLC-pRcCMV.

3. Co-expression of CD4-IgG2HC-pRcCMV and CD4-kLC-pRcCMV in mammalian cells to produce CD4-IgG2 chimeric heterotetramer.

a. Transient expression.

CosM5 cells grown in DMEM containing 10% fetal calf serum are split to 75% confluence. On the following day, the cells are transfected for 16-20 hours with 5 micrograms of CsCl purified CD4-IgG2HC-pRcCMV DNA and 5 micrograms of CsCl-purified CD4-kLC-pRcCMV plasmid DNA by the standard CaPO(4) precipitation technique. After transfection, fresh medium is added to the cells. Analysis of the products synthesized 48-72 hours post-transfection is performed by radiolabelling of transfectants with ^{35}S-methionine for 12-18 hours followed by precipitation of media and cell lysates using anti-CD4 antibodies or by incubation with Protein A-sepharose beads alone followed by SDS-PAGE under reducing or non-reducing conditions. In addition, analysis of media and cell lysates is performed 48-72 hours post-transfection by standard Western blotting procedures.

b. Stable expression.

Dhfr-Chinese hamster ovary cells (CHO) are transfected with 20 micrograms of CsCl purified DNA in a ratio of 1000:1000:1 CD4-IgG2HC-pRcCMV:CD4-kLC-pRcCMV:p410 (p410 is an expression plasmid containing the dhfr gene), although other ratios may

also be used. At approximately 3-5 days post-transfection, cells are placed in selective medium (nucleoside-free alpha MEM containing 10% dialyzed fetal calf serum). At approximately 10-15 days post-selection, individual cell clones are picked. The clones are then analyzed for stable expression of CD4-IgG2 chimeric heterotetramers by several screening techniques, such as ELISA and precipitation with Protein A-sepharose beads followed by SDS-PAGE under reducing or non-reducing conditions. Clones expressing the highest levels are subjected to successive rounds of amplification of the newly introduced DNA sequences in increasing concentrations of methotrexate. Stable CHO cell lines are thus generated which secrete high levels of CD4-IgG2 chimeric heterotetramer.

4. Purification of CD4-IgG2 chimeric heterotetramers from CHO conditioned media:

CD4-IgG2 chimeric heterotetramers are purified using Protein A-Sepharose column chromatography. CHO cells secreting CD4-IgG2 chimeric heterotetramers are grown to high density in roller bottles in medium containing alpha MEM with 10% IgG-free fetal calf serum. Conditioned media is collected, clarified by centrifugation, and diluted 1:1 with PBS with/or without detergent (i.e. Tween) in this and subsequent buffers. The diluted media is then applied to a 5ml column of Protein A-Sepharose fast flow previously equilibrated with PBS, at a flow rate of 60ml/hour. After extensive washing, the bound material is eluted with 100mM glycine/HCl, pH 3.5, directly into an aliquot of 1M Tris.HCl pH 8.0 to immediately neutralize the eluted fractions. Fractions are then analyzed by SDS-PAGE under reducing and non-reducing conditions followed by silver staining and pooled (Figure 8).

5. Demonstration of binding of CD4-IgG2 chimeric

heterotetramer to the envelope glycoprotein gp120:

CosM5 transfectants expressing CD4-IgG2 chimeric heterotetramers are incubated for 72 hours in DMEM containing 10% IgG-free fetal calf serum. Unlabelled medium is then collected and used to precipitate ^{35}S-methionine-radiolabelled HIV gp120. After incubation of CD4-IgG2 chimeric heterotetramer containing medium with ^{35}S-methionine-labelled gp120, the complexes are adsorbed to Protein A-sepharose. Protein A-sepharose complexes are recovered by centrifugation, and the precipitates are analyzed by SDS-PAGE followed by fluorography. Alternatively, aliquots of purified CD4-IgG2 chimeric heterotetramers from CHO cells are also used to precipitate ^{35}S-radiolabelled gp120 using the same procedure.

6. Determination of plasma half-life and placental transfer of CD4-IgG2 chimeric heterotetramer:

Determination of the plasma half-life and placental transfer are performed by well established techniques. Briefly, rabbits or monkeys are injected intravenously or intramuscularly with purified CD4-IgG2 chimeric heterotetramer. At various time points post-injection, plasma samples are taken, and the quantity of the CD4-IgG2 chimeric heterotetramer present in the serum is measured by ELISA. In addition, pregnant monkeys are also injected either IV or IM with CD4-IgG2 chimeric heterotetramer and the concentration determined in the cord blood and the serum of the newborn monkey. Determination and comparison of the quantity of the CD4-IgG2 chimeric heterotetramer in the mother's serum as well as in the cord blood and serum of the newborn indicates the relative rate of transport across the placenta of these molecules.

7. Determination of FcR binding and macrophage infectivity of CD4-IgG2 chimeric heterotetramer:

Determination of FcR binding and macrophage infectivity of CD4-IgG2 chimeric heterotetramer are performed by well established techniques. For these studies, U937 cells (a human monocytic cell line expressing FcR1 and FcRII), purified monocyte/macrophage populations from human peripheral blood, and Hela cells constitutively expressing recombinant human FcRs are utilized. In addition, monoclonal antibodies specific for FcR1 and FcRII are commercially available. Briefly, radiolabelled monomeric or aggregated CD4-IgG2 chimeric heterotetramer is incubated with the above cells and appropriate control cells at 4 degrees Celsius over various time points. At the end of each incubation, the cells are solubized and the cell-associated radioactivity is determined to establish the amount of CD4-IgG2 chimeric heterotetramer specifically bound to each cell type. As controls, radiolabelled normal monomeric or aggregated human IgG2 are used to determine the levels of specific antibody binding. Furthermore, competition of the radiolabelled component with unlabelled monomeric or aggregated normal human IgG2, or monoclonal antibodies to FcRI or FcRII, will establish the binding efficiency and specificity of CD4-IgG2 chimeric heterotetramer to each cell type.

To ascertain whether the CD4-IgG2 chimeric heterotetramer mediates enhancement of HIV infection of monocytes/macrophages, HIV-1 is incubated with media alone or either monomeric or aggregated CD4-IgG2 chimeric heterotetramer at several dilutions. As controls, sera from normal individuals and HIV-infected individuals are used (31). After incubation for one hour at 4 degrees Celsius, the 'opsonized' virus is added to the cell types described in the paragraph above. At various time points after

infection, the media is harvested and assayed for viral reverse transcriptase activity to determine the degree of viral infection. As controls, sCD4, OKT4a or Leu3a are included during the infection of the cells. In addition, various dilutions of the CD4-IgG2 chimeric heterotetramer and appropriate controls are incubated first with the cells at 4 degress Celsius to allow binding. HIV is then added and infection assayed by viral reverse transcriptase activity.

B. Results

A CD4-gamma2 chimeric heavy chain gene encoding a CD4-gamma2 chimeric heavy chain homodimer was generated by ligating the leader-V1-V2 segment of the human CD4 cDNA (4) to the hinge exon of the human gamma2 heavy chain gene (30) (Figure 1A). The resulting recombinant DNA molecule (designated CD4-IgG2-Rf) encodes the signal sequence and two amino-terminal immunoglobulin-like domains of the CD4 protein (the first 179 amino acids of mature CD4) followed by the hinge (15 amino acids), CH2 (110 amino acids), and CH3 (107 amino acids) regions of the gamma2 heavy chain protein (Figure 3). This recombinant DNA molecule also contains two introns present within the gamma2 heavy chain gene: between the H and CH2 domains, and between the CH2 and CH3 domains. This CD4-gamma2 chimeric gene was designed to encode a CD4-gamma2 chimeric heavy chain homodimer which specifically lacks the CH1 domain of the gamma2 heavy chain. Expression of the CH1 domain without accompanying light chains prevents efficient heavy chain secretion from mammalian cells (25).

In the CD4-gamma2 chimeric heavy chain homodimer, the hinge region of one chain contains four cysteine residues, affording the potential of four interchain disulfide bonds (Figure 1B). Similarly, naturally-occuring human IgG2 contains four interchain disulphide bonds between the gamma2

heavy chains.

The CD4-gamma2 chimeric heavy chain gene was subcloned into the mammalian expression vector pcDNA1. This vector contains the following DNA elements: the cytomegalovirus (CMV) immediate early promoter and enhancer driving transcription of the CD4-gamma2 chimeric heavy chain gene; an SV40 polyadenylation sequence; and an SV40 origin of replication which allows replication of the plasmid to high copy number in CosM5 cells. The resulting CD4-gamma2 heavy chain mammalian expression vector (designated CD4-IgG2-pcDNA1) was transfected into CosM5 cells which were then radiolabelled with ^{35}S-methionine 48-72 hours post-transfection. The radiolabelled medium was analyzed by precipitation with Protein A-sepharose beads and SDS-PAGE followed by fluorography (Figure 6). Under reducing conditions, a protein migrating at a relative molecular mass (Mr) of approximately 47 kilodaltons is precipitated. When the precipitated material was run on SDS-PAGE under nonreducing conditions, a protein migrating at an Mr of approximately 94 kilodaltons is observed, indicating that the CD4-gamma2 chimeric heavy chains assemble and are secreted as homodimers. In addition, these results demonstrate that the secreted CD4-gamma2 chimeric heavy chain homodimers contain an intact immunoglobulin Fc domain since they bind Protein A. Further characterization by Western blot analysis of the proteins secreted into the medium 48-72 hours post-transfection was performed using a rabbit polyclonal antiserum raised against purified soluble human CD4. Similar to the results obtained by precipitation, when the medium was run on SDS-PAGE under reducing conditions, followed by Western transfer to nitrocellulose, the major immunoreactive protein migrates at an Mr of approximately 47 kilodaltons. Under nonreducing conditions, the major immunoreactive protein migrates at an Mr of approximately 94 kilodaltons. Taken together, these

results demonstrate that the CD4-gamma2 chimeric heavy chain is produced and secreted as a homodimer of the predicted molecular weight.

The above results demonstrate that the Fc portion of CD4-gamma2 chimeric heavy chain homodimer, encoded by the constant regions of the gamma2 heavy chain gene, binds Protein A and is therefore functionally active. In order to determine if the CD4 portion is functionally intact, CD4-gamma2 chimeric heavy chain homodimers were assayed for their ability to bind to the HIV exterior envelope glycoprotein, gp120 (Figure 7). Unlabelled medium from CosM5 cells transfected with CD4-IgG2-pcDNA1 DNA was incubated with ^{35}S-methionine-labelled gp120. CD4-gamma2 chimeric heavy chain homodimer/gp120 complexes were precipitated by incubation with Protein A-sepharose beads, and the precipitates were analyzed by SDS-PAGE under reducing conditions followed by fluorography. These results demonstrate that the CD4-gamma2 chimeric heavy chain homodimer efficiently recognizes HIV gp120 and binds with high affinity. These observations, taken together with the results described in the above paragraph, demonstrate that CD4-gamma2 chimeric heavy chain homodimer contains functionally active regions of both CD4 and gamma2 heavy chain.

In order to stably produce large quantities of the CD4-gamma2 chimeric heavy chain homodimers, the CD4-IgG2-pcDNA1 vector was cotransfected with the plasmid p410 (encoding the enzyme dihydrofolate reductase (dhfr)) into dhfr-Chinese Hamster Ovary (CHO) cells. Approximately two weeks post-transfection, individual clones growing in nucleoside free alpha MEM and 10% dialyzed fetal calf serum (and therefore dhfr+) were isolated and analyzed for co-expression of CD4-gamma2 chimeric heavy chain homodimers by precipitation and ELISA. The highest producing cell lines were identified and

subjected to stepwise increasing concentrations of methotrexate which selects for amplification of the newly introduced DNA sequences. A CHO cell line expressing 10 micrograms/milliliter of CD4-gamma2 chimeric heavy chain homodimer was used for stable, constitutive production in roller bottles. The cells were grown to confluence in alpha MEM containing 10% IgG-free fetal calf serum. The cells were then fed every other day and two day old conditioned medium was used for purification of the CD4-gamma2 chimeric heavy chain homodimer. Conditioned medium was diluted 1:1 with phosphate-buffered saline (PBS) and applied to a 5ml column of Protein A-sepharose fast flow (Pharmacia) at a flow rate of 60 milliliters/hour. The column was then washed with 10 column volumes of PBS and the bound material was eluted with 100 mM glycine pH 3.5. The eluted material was collected directly into 50μl of 1M Tris. HCl pH 8.0 to neutralize the eluant. Fractions having an OD(280) of greater than 0.1 were analyzed by SDS-PAGE followed by silver staining or Western blot analysis, and the peak fractions were pooled. A single band was specifically eluted from the Protein A-sepharose column with an Mr corresponding to the CD4-gamma2 chimeric heavy chain homodimer (Figure 8). Western blot analysis confirms that the eluted protein is immunoreactive with polyclonal antiserum raised against soluble human CD4. In addition, the purified protein retains the ability to bind with high affinity to ^{35}S-methionine-labelled gp120. These results demonstrate the stable, high-level production of CD4-gamma2 chimeric heavy chain homodimers in mammalian cells, and the purification of CD4-gamma2 chimeric heavy chain homodimer which retains biological function.

The partially purified CD4-gamma2 heavy chain homodimer purified as described in Figure 8 was effective at preventing HIV binding to CD4 cells (Figure 9) and neutralization of infectivity of a fixed HIV inoculum

(Figure 10). In this later assay, approximately 10-25 μg/ml of CD4-gamma2 as well as sCD4 were required to prevent 50% of the cultures from becoming infected by HIV.

Further purification of CD4-gamma2 heavy chain homodimer was achieved using ion-exchange chromatography. The peak fraction from the protein A-sepharose column was applied to a 10ml S-sepharose fast flow column preequilibrated with 50mM BES pH 7.0, at a flow rate of 120ml/hr. After application of the sample, the column was extensively washed with 50mM BES pH 7.0 with increasing salt concentration (see materials and methods). CD4-gamma2 heavy chain homodimer was specifically eluted from the column in 50mM BES pH 7.0 containing 500mM NaCl. Following the ion exchange chromatography, we unexpectedly found the peak fractions containing the CD4-gamma2 chimeric heavy chain homodimer was still impure. Therefore, the peak fractions from the S-sepharose column were pooled, concentrated and applied to a 120ml Sephacryl S-300HR column preequilibrated with PBS and run at a flow rate of 8 ml per hour. The peak fractions of purified CD4-gamma2 heavy chain homodimer were analyzed by SDS-PAGE and silver staining under non-reducing conditions, and the purified fractions were pooled and analyzed by SDS-PAGE followed by silver staining under non-reducing conditions (Figure 11, lane 1), or reducing conditions (Figure 11, lane 2). When the purified CD4-gamma2 chimeric heavy chain homodimer was run on SDS-PAGE under reducing conditions, a doublet was observed which appeared to be due to differences in glycosylation of the CD4-gamma2 chimeric heavy chain homodimer (data not shown).

A CD4-IgG2HC chimeric heavy chain gene encoding a CD4-IgG2 chimeric heavy chain was generated by ligating the leader-V1-V2 segment of the human CD4 cDNA to the CH1 exon of the human IgG2 heavy chain gene (Figure 2A). In addition a CD4-kappa chimeric light chain gene encoding a CD4-kappa light

chain was generated by ligating the leader-V1-V2 segment of the human CD4 cDNA to the constant domain of the kappa light chain gene (Figure 2A). These CD4-IgG2 chimeric heavy chain genes and CD4-kappa chimeric light chain genes were designed to encode a CD4-IgG2 chimeric heterotetramer, in which the CD4-IgG2 heavy chain contains a CH1 domain for efficient association with kappa light chains.

Both the CD4-IgG2 chimeric heavy chain and the CD4-kappa chimeric light chain genes were subcloned into the mammalian expression vectors pRcCMV or pPPI-2. Both vectors contain the cytomegalovirus immediate early promoter and enhancer driving transcription of the chimeric genes. In the vector pRcCMV, a second transcriptional cassette which contains the RSV promoter and enhancer is used to direct the transcription of the neomycin resistance gene. In pPPI-2, a second transcriptional cassette which contains the ß-globin promoter directs the transcription of the dhfr gene (see supra). In order to stably produce large quantities of the CD4-IgG2 chimeric heterotetramer, the CD4-IgG2 chimeric heavy chain expression vector and the CD4-kappa chimeric light chain expression vector were transfected simultaneously (typically the CD4-IgG2 chimeric heavy chain gene cloned in pRcCMV was used, and CD4-kappa chimeric light chain gene cloned in pPPI-2 was used in a ratio of 1:1). Approximately two weeks post-transfection, individual clones growing in nucleoside-free alpha MEM containing 1 mg/ml G418 and 10% dialyzed fetal calf serum were isolated and analyzed for co-expression of both CD4-IgG2 chimeric heavy chains and CD4-kappa chimeric light chains by immunoprecipitation and ELISA. Figure 12 demonstrates one clone which was selected and analyzed for the expression of both CD4-IgG2 chimeric heavy chains and CD4-kappa chimeric light chains. The CHO cell line or the untransfected parental CHO cell line were radiolabelled with ^{35}S-methionine and ^{35}S-cysteine for 16 hours. The radiolabelled medium was analyzed by

precipitation with Protein A-sepharose beads and SDS-PAGE under non-reducing conditions followed by fluorography (Figure 12A). Under non-reducing conditions 2 proteins migrating at relative molecular masses of approximately 140 kilodaltons and 210 kilodaltons are precipitated. When the precipitated material was run on SDS-PAGE under non-reducing conditions, 2 proteins migrating at relative molecular masses of 69 kilodaltons and 35 kilodaltons were observed, which are consistent with the relative predicted molecular masses of the CD4-IgG2 chimeric heavy chains, and CD4-kappa chimeric light chains respectively (data now shown). Further characterization has shown that the protein migrating at 210 kilodaltons on SDS-PAGE under non-reducing conditions contains both CD4-IgG2 chimeric heavy chains and CD4-kappa chimeric light chains which are covalently associated, while the protein migrating at 140 kilodaltons on SDS-PAGE under non-reducing conditions contains only CD4-IgG2 chimeric heavy chains (Figure 12B). These data are consistent with the predicted molecular weight of the 210 kilodalton protein having 2 CD4-IgG2 chimeric heavy chains and 2 CD4-kappa chimeric light chains, covalently associated to form a molecule with the structure H_2L_2 (H=heavy chain, L-light chain). Furthermore, the 140 kilodalton protein seen on SDS-PAGE under non-reducing conditions is consistent with the predicted molecular weight of a CD4-IgG2 chimeric homodimer having the structure H_2. Taken together, these results indicate that a CHO cell line which expresses both CD4-IgG2 chimeric heavy chains and CD4-kappa chimeric light chains is able to efficiently assemble and secrete CD4-IgG2 chimeric heterotetramers.

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What is claimed is :

1. An expression vector encoding a CD4-gamma2 chimeric heavy chain homodimer designated CD4-IgG2-pcDNA1 (ATCC No. 40952).

2. A CD4-gamma2 chimeric heavy chain homodimer encoded by the expression vector of claim 1.

3. A method of producing a CD4-gamma2 chimeric heavy chain homodimer which comprises:

   a) transfecting a mammalian cell with the expression vector of claim 1;

   b) culturing the resulting transfected mammalian cell under conditions such that chimeric heavy chain homodimer is produced; and

   c) recovering the chimeric heavy chain homodimer so produced.

4. A method of claim 3, wherein the mammalian cell is a COS cell, CHO cell or myeloma cell.

5. A method of inhibiting HIV infection of a CD4+ cell which comprises treating the CD4+ cell with an amount of the CD4-gamma2 chimeric heavy chain homodimer of claim 2 effective to inhibit infection of the cell.

6. A method of preventing a subject from being infected with HIV which comprises administering to the subject an amount of the CD4-gamma2 chimeric heavy chain homodimer of claim 2 effective to prevent the subject from being infected with HIV.

7. A method of treating a subject infected with HIV so as to block the spread of HIV infection which comprises administering to the subject an amount of the CD4-gamma2 chimeric heavy chain homodimer of claim 2 effective to block the spread of HIV infection.

8. A pharmaceutical composition which comprises the CD4-gamma2 chimeric heavy chain homodimer of claim 2 in an amount effective to inhibit HIV infection of a CD4+ cell and a pharmaceutically acceptable carrier.

9. A composition of matter comprising a CD4-gamma2 chimeric heavy chain homodimer of claim 2 and a toxin linked thereto.

10. A composition of claim 9, wherein the toxin is the deglycosylated A chain of ricin, domains II or III of Pseudomonas exotoxin A, or Diphtheria toxin.

11. A diagnostic reagent comprising a CD4-gamma2 chimeric heavy chain homodimer of claim 2 and a detectable marker linked thereto.

12. A diagnostic reagent of claim 11 wherein the detectable marker is a radioisotope, chromophore, or fluorophore.

13. An expression vector encoding the heavy chains of a CD4-IgG2 chimeric heterotetramer designated CD4-IgG2HC-pRcCMV (ATCC No. 75193).

14. An expression vector encoding the light chains of a CD4-IgG2 chimeric heterotetramer designated CD4-kLC-pRcCMV (ATCC No. 75194).

15. A CD4-IgG2 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector of claim 13.

16. A CD4-IgG2 chimeric heterotetramer, the light chains of which are encoded by the expression vector of claim 14.

17. A CD4-IgG2 chimeric heterotetramer the heavy and the light chains of which are encoded by the expression vectors of claims 13 and 14, respectively.

18. A method of producing a CD4-IgG2 chimeric heterotetramer which comprises:

a) cotransfecting a mammalian cell with the expression vector of claim 13 and an expression vector encoding a light chain;

b) culturing the resulting cotransfected mammalian cell under conditions such that the CD4-IgG2 chimeric heterotetramer is produced; and

c) recovering the CD4-IgG2 chimeric heterotetramer so produced.

19. A method of producing an CD4-IgG2 chimeric heterotetramer which comprises:

a) cotransfecting a mammalian cell with the expression vector of claim 14 and an expression vector encoding an IgG2 heavy chain and;

b) culturing the resulting cotransfected mammalian cell under conditions such that the chimeric heterotetramer is produced; and

c) recovering the chimeric heterotetramer so produced.

20. A method of producing a CD4-IgG2 chimeric heterotetramer which comprises:

a) cotransfecting a mammalian cell with the expression vectors of claim 13 and 14;

b) culturing the resulting cotransfected mammalian cell under conditions such that the chimeric heterotetramer is produced; and

c) recovering the chimeric heterotetramer so produced.

21. A method of claim 18, 19 or 20, wherein the mammalian cell is a COS cell, CHO cell or myeloma cell.

22. A method of inhibiting HIV infection of a CD4+ cell which comprises treating the CD4+ cell with an amount of the CD4-IgG2 chimeric heterotetramer of claim 15, 16 or 17 effective to inhibit infection of the cell.

23. A method of preventing a subject from being infected with HIV which comprises administering to the subject an amount of the CD4-IgG2 chimeric heterotetramer of claim 15, 16 or 17 effective to prevent the subject from being infected with HIV.

24. A method of treating a subject infected with HIV so as to block the spread of HIV infection which comprises administering to the subject an amount of CD4-IgG2 chimeric heterotetramer of claim 15, 16 or 17 effective to block spread of HIV infection.

25. A pharmaceutical composition which comprises the CD4-IgG2 chimeric heterotetramer of claim 15, 16 or 17 in an amount effective to inhibit HIV infection of a CD4+ cell and a pharmaceutically acceptable carrier.

26. A composition of matter comprising a CD4-IgG2 chimeric heterotetramer of claim 15, 16 or 17 and a toxin linked thereto.

27. A composition of claim 26, wherein the toxin is the deglycosylated A chain of ricin, domains II or III of Pseudomonas exotoxin A, and Diphtheria toxin.

28. A diagnostic reagent comprising a CD4-IgG2 chimeric heterotetramer of claim 15, 16 or 17 and a detectable marker linked thereto.

29. A diagnostic reagent of claim 28 wherein the detectable marker is a radioisotope, chromophore or fluorophore.

add E2

CD4-Gamma2 AND CD4-IgG2 CHIMERAS

Abstract of the Invention

This invention provides an expression vector encoding a CD4-gamma2 chimeric heavy chain homodimer. This invention also provides an expression vector encoding the heavy chains of a CD4-IgG2 chimeric heterotetramer. Finally, this invention provides an expression vector encoding the light chains of a CD4-IgG2 chimeric heterotetramer.

Figure 1A

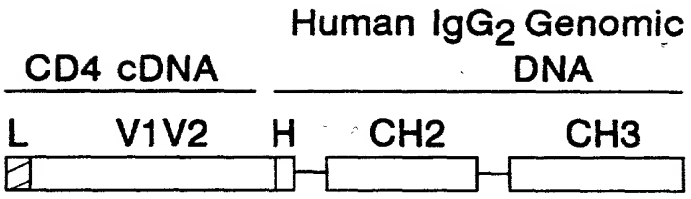


Figure 1B

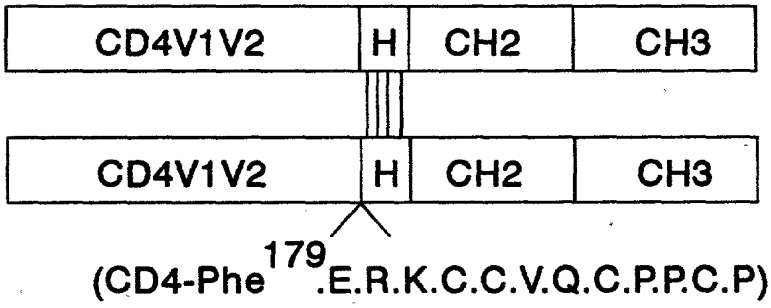
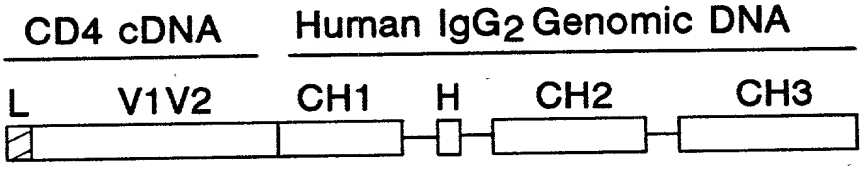


Figure 2A



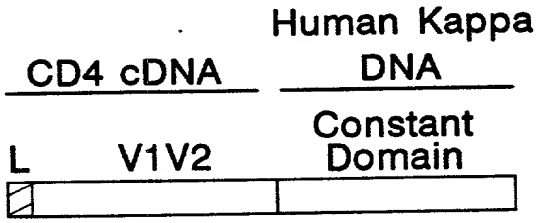


Figure 2B

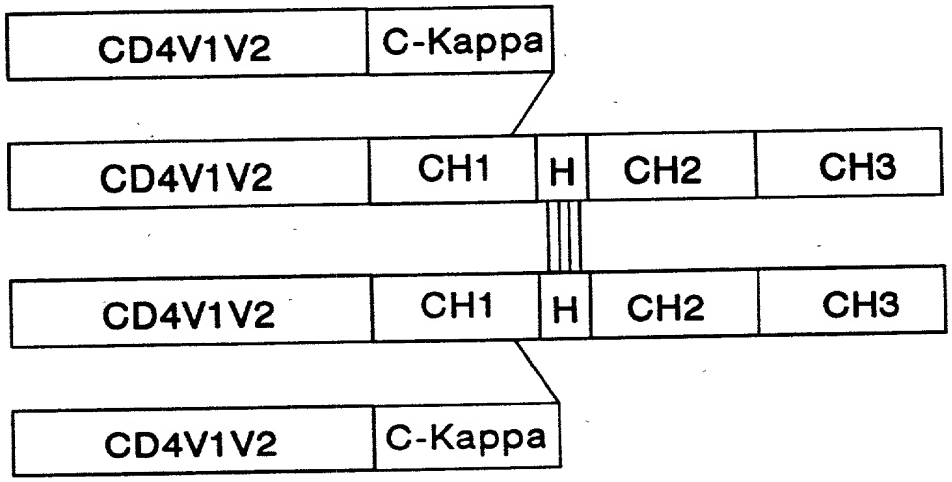


FIGURE 3

```
CAAGCCCAGAGCCCTGCCATTTCTGTGGGCTCAGGTCCCTACTGCTCAGCCCCTT      55
                         ┌─>CD4
                         │                     -20
                         M   N   R   G   V   P   F   R   H
CCTCCCTCGGCAAGGCCACAATG AAC CGG GGA GTC CCT TTT AGG CAC     102

                            -10
 L   L   L   V   L   Q   L   A   L   L   P   A   A   T
TTG CTT CTG GTG CTG CAA CTG GCG CTC CTC CCA GCA GCC ACT     144

    -1  +1                                   +10
 Q   G   K   K   V   V   L   G   K   K   G   D   T   V
CAG GGA AAG AAA GTG GTG CTG GGC AAA AAA GGG GAT ACA GTG     186

                            +20
 E   L   T   C   T   A   S   Q   K   K   S   I   Q   F
GAA CTG ACC TGT ACA GCT TCC CAG AAG AAG AGC ATA CAA TTC     228

            +30                                     +40
 H   W   K   N   S   N   Q   I   K   I   L   G   N   Q
CAC TGG AAA AAC TCC AAC CAG ATA AAG ATT CTG GGA AAT CAG     270

                                    +50
 G   S   F   L   T   K   G   P   S   K   L   N   D   R
GGC TCC TTC TTA ACT AAA GGT CCA TCC AAG CTG AAT GAT CGC     312
```

```
 G   S   F   L   T   K   G   P   S   K   L   N   D   R
GGC TCC TTC TTA ACT AAA GGT CCA TCC AAG CTG AAT GAT CGC      312

                    +60
 A   D   S   R   R   S   L   W   D   Q   G   N   F   P
GCT GAC TCA AGA AGA AGC CTT TGG GAC CAA GGA AAC TTC CCC      354

    +70                                     +80
 L   I   I   K   N   L   K   I   E   D   S   D   T   Y
CTG ATC ATC AAG AAT CTT AAG ATA GAA GAC TCA GAT ACT TAC      396

                            +90
 I   C   E   V   E   D   Q   K   E   E   V   Q   L   L
ATC TGT GAA GTG GAG GAC CAG AAG GAG GAG GTG CAA TTG CTA      438

            +100                                    +110
 V   F   G   L   T   A   N   S   D   T   H   L   L   Q
GTG TTC GGA TTG ACT GCC AAC TCT GAC ACC CAC CTG CTT CAG      480

                                    +120
 G   Q   S   L   T   L   T   L   E   S   P   P   G   S
GGG CAG AGC CTG ACC CTG ACC TTG GAG AGC CCC CCT GGT AGT      522

                    +130
 S   P   S   V   Q   C   R   S   P   R   G   K   N   I
AGC CCC TCA GTG CAA TGT AGG AGT CCA AGG GGT AAA AAC ATA      564
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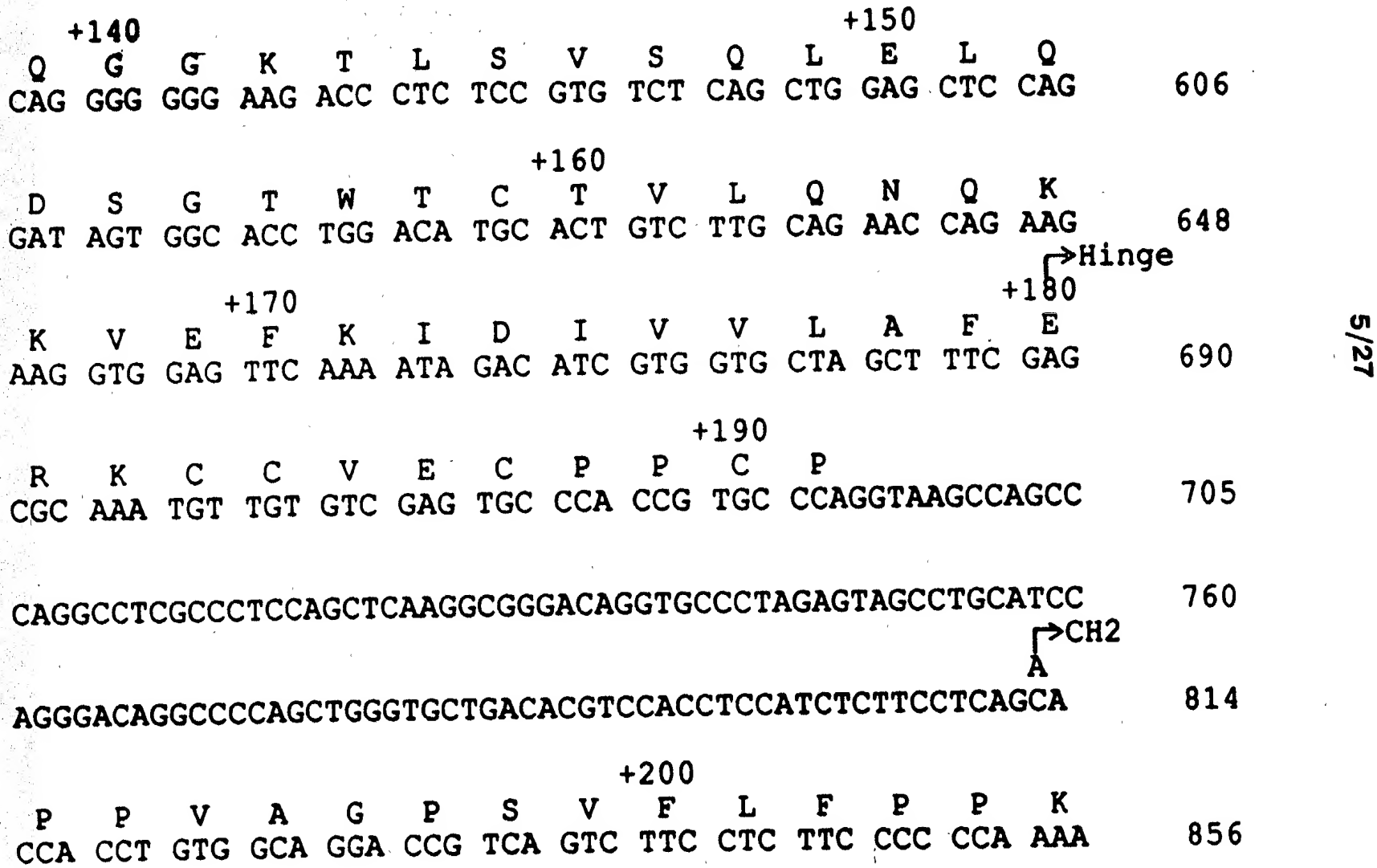

```
                  +210
P   K   D   T   L   M   I   S   R   T   P   E   V   T
CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACG      898

+220                                        +230
C   V   V   V   D   V   S   H   E   D   P   E   V   Q
TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCC GAG GTC CAG      940

                        +240
F   N   W   Y   V   D   G   V   E   V   H   N   A   K
TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG      982

        +250                                    +260
T   K   P   R   E   E   Q   F   N   S   T   F   R   V
ACA AAG CCA CGG GAG GAG CAG TTC AAC AGC ACG TTC CGT GTG      1024

                                +270
V   S   V   L   T   V   V   H   Q   D   W   L   N   G
GTC AGC GTC CTC ACC GTT GTG CAC CAG GAC TGG CTG AAC GGC      1066

                  +280
K   E   Y   K   C   K   V   S   N   K   G   L   P   A
AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GGC CTC CCA GCC      1108

+290
P   I   E   K   T   I   S   K   T   K
CCC ATC GAG AAA ACC ATC TCC AAA ACC AAAGGTGGGACCCGCGGGG      1154
```

```
TATGAGGGCCACATGGACAGAGGCCGGCTCGGCCCACCCTCTGCCCTGGGAGTGA          1209
                                      ┌→CH3
                                    +300
                                      G   Q   P   R.  E   P   Q
CCGCTGTGCCAACCTCTGTCCCTACAGGG CAG CCC CGA GAA CCA CAG            1256

            +310                                          +320
 V   Y   T   L   P   P   S   R   E   E   M   T   K   N
GTG TAC ACC CTG CCC CCA TCC CGG GAG GAG ATG ACC AAG AAC          1298

                                     +330
 Q   V   S   L   T   C   L   V   K   G   F   Y   P   S
CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAC CCC AGC          1340

                        +340
 D   I   A   V   E   W   E   S   N   G   Q   P   E   N
GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC          1382

    +350                                     +360
 N   Y   K   T   T   P   P   M   L   D   S   D   G   S
AAC TAC AAG ACC ACA CCT CCC ATG CTG GAC TCC GAC GGC TCC          1424

                            +370
 F   F   L   Y   S   K   L   T   V   D   K   S   R   W
TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG          1466
```

```
                +380                                                +400
 Q   Q   G   N   V   F   S   C   S   V   M   H   E   A
CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT      1508

                                    +410
 L   H   N   H   Y   T   Q   K   S   L   S   L   S   P
CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG      1550

 G   K  stop
GGT AAA TGAGTGCCACGGCCGGCAAGCCCCCGCTCCCCAGGCTCTCGGGGTCG      1603

CGTGAGGATGCTTGGCACGTACCCCGTGTACATACTTCCCAGGCACCCAGCATGG      1658

AAATAAAGCACCCAGCGCTGCCCTGGGCCCCTGCGAGACTGTGATGGTTCTTTCC      1713

GTGGGTCAGGCCGAGTCTGAGGCCTGAGTGGCATGAGGGAGGCAGAGTGGGTC...    1766
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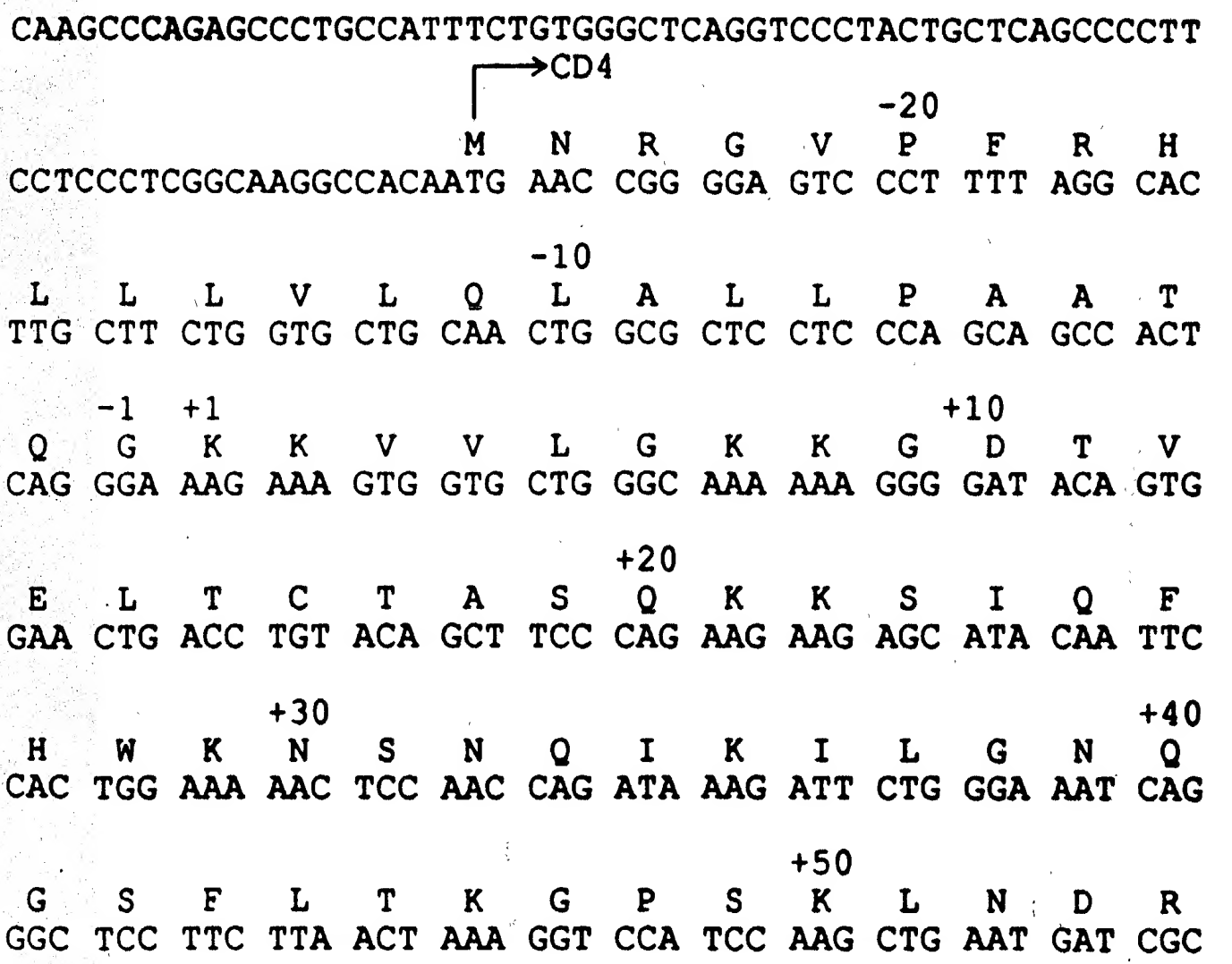
FIGURE 4



9/27 FIGURE 4
10/27
11/27
12/17
13/27
14/27
15/27
16/27

9/27

PCT/US96/01143

07/960,440

```
                         +60
 A   D   S   R   R   S   L   W   D   Q   G   N   F   P
GCT GAC TCA AGA AGA AGC CTT TGG GAC CAA GGA AAC TTC CCC      354

    +70                                     +80
 L   I   I   K   N   L   K   I   E   D   S   D   T   Y
CTG ATC ATC AAG AAT CTT AAG ATA GAA GAC TCA GAT ACT TAC      396

                             +90
 I   C   E   V   E   D   Q   K   E   E   V   Q   L   L
ATC TGT GAA GTG GAG GAC CAG AAG GAG GAG GTG CAA TTG CTA      438

            +100                                    +110
 V   F   G   L   T   A   N   S   D   T   H   L   L   Q
GTG TTC GGA TTG ACT GCC AAC TCT GAC ACC CAC CTG CTT CAG      480

                                    +120
 G   Q   S   L   T   L   T   L   E   S   P   P   G   S
GGG CAG AGC CTG ACC CTG ACC TTG GAG AGC CCC CCT GGT AGT      522

                    +130
 S   P   S   V   Q   C   R   S   P   R   G   K   N   I
AGC CCC TCA GTG CAA TGT AGG AGT CCA AGG GGT AAA AAC ATA      564
```

~~#7/960440~~ 07/960,440


```
   +140                                          +150
 Q   G   G   K   T   L   S   V   S   Q   L   E   L   Q
CAG GGG GGG AAG ACC CTC TCC GTG TCT CAG CTG GAG CTC CAG      606

                             +160
 D   S   G   T   W   T   C   T   V   L   Q   N   Q   K
GAT AGT GGC ACC TGG ACA TGC ACT GTC TTG CAG AAC CAG AAG      648
                                                     ┌→CH1
            +170                                   +180
 K   V   E   F   K   I   D   I   V   V   L   A   F   A
AAG GTG GAG TTC AAA ATA GAC ATC GTG GTG CTA GCT TTC GCC      690

                                     +190
 S   T   K   G   P   S   V   F   P   L   A   P   C   S
TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCG CCC TGC TCC      732

                    +200
 R   S   T   S   E   S   T   A   A   L   G   C   L   V
AGG AGC ACC TCC GAG AGC ACA GCC GCC CTG GGC TGC CTG GTC      774

   +210                                          +220
 K   D   Y   F   P   E   P   V   T   V   S   W   N   S
AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA      816

                             +230
 G   A   L   T   S   G   V   H   T   F   P   A   V   L
GGC GCT CTG ACC AGC GGC GTG CAC ACC TTC CCA GCT GTC CTA      858
```

```
                    +240                                                  +250
 Q    S    S    G    L    Y    S    L    S    S    V    V    T    V
CAG  TCC  TCA  GGA  CTC  TAC  TCC  CTC  AGC  AGC  GTG  GTG  ACC  GTG      900

                                                 +260
 P    S    S    N    F    G    T    Q    T    Y    T    C    N    V
CCC  TCC  AGC  AAC  TTC  GGC  ACC  CAG  ACC  TAC  ACC  TGC  AAC  GTA      942

                              +270
 D    H    K    P    S    N    T    K    V    D    K    T    V
GAT  CAC  AAG  CCC  AGC  AAC  ACC  AAG  GTG  GAC  AAG  ACA  GTTGGTG       985

AGAGGCCAGCTCAGGGAGGGAGGGTGTCTGCTGGAAGCCAGGCTCAGCCCTCCTG      1040

CCTGGACGCACCCCGGCTGTGCAGCCCCAGCCCAGGGCAGCAAGGCAGGCCCCAT      1095

CTGTCTCCTCACCCGGAGGCCTCTGCCCGCCCCACTCATGCTCAGGGAGAGGGTC      1150

TTCTGGCTTTTTCCACCAGGCTCCAGGCAGGCACAGGCTGGGTGCCCCTACCCCA      1205

GGCCCTTCACACACAGGGGCAGGTGCTTGGCTCAGACCTGCCAAAAGCCATATCC      1260
```

```
GGGAGGACCCTGCCCCTGACCTAAGCCGACCCCAAAGGCCAAACTGTCCACTCCC      1315

TCAGCTCGGACACCTTCTCTCCTCCCAGATCCGAGTAACTCCCAATCTTCTCTCT      1370
  ┌→Hinge
  |    +280
  E     R   K   C   C   V   E   C   P   P   C   P
GCAGAG CGC AAA TGT TGT GTC GAG TGC CCA CCG TGC CCAGGTAAG     1415

CCAGCCCAGGCCTCGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAGTAGCCT      1470

GCATCCAGGGACAGGCCCCAGCTGGGTGCTGACACGTCCACCTCCATCTCTTCCT      1525
   ┌→CH2
 +290                                        +300
   A    P   P   V   A   G   P   S   V   F   L   F   P   P
CAGCA CCA CCT GTG GCA GGA CCG TCA GTC TTC CTC TTC CCC CCA    1569

                      +310
 K   P   K   D   T   L   M   I   S   R   T   P   E   V
AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC      1611

         +320                                  +330
 T   C   V   V   V   D   V   S   H   E   D   P   E   V
ACG TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCC GAG GTC      1653
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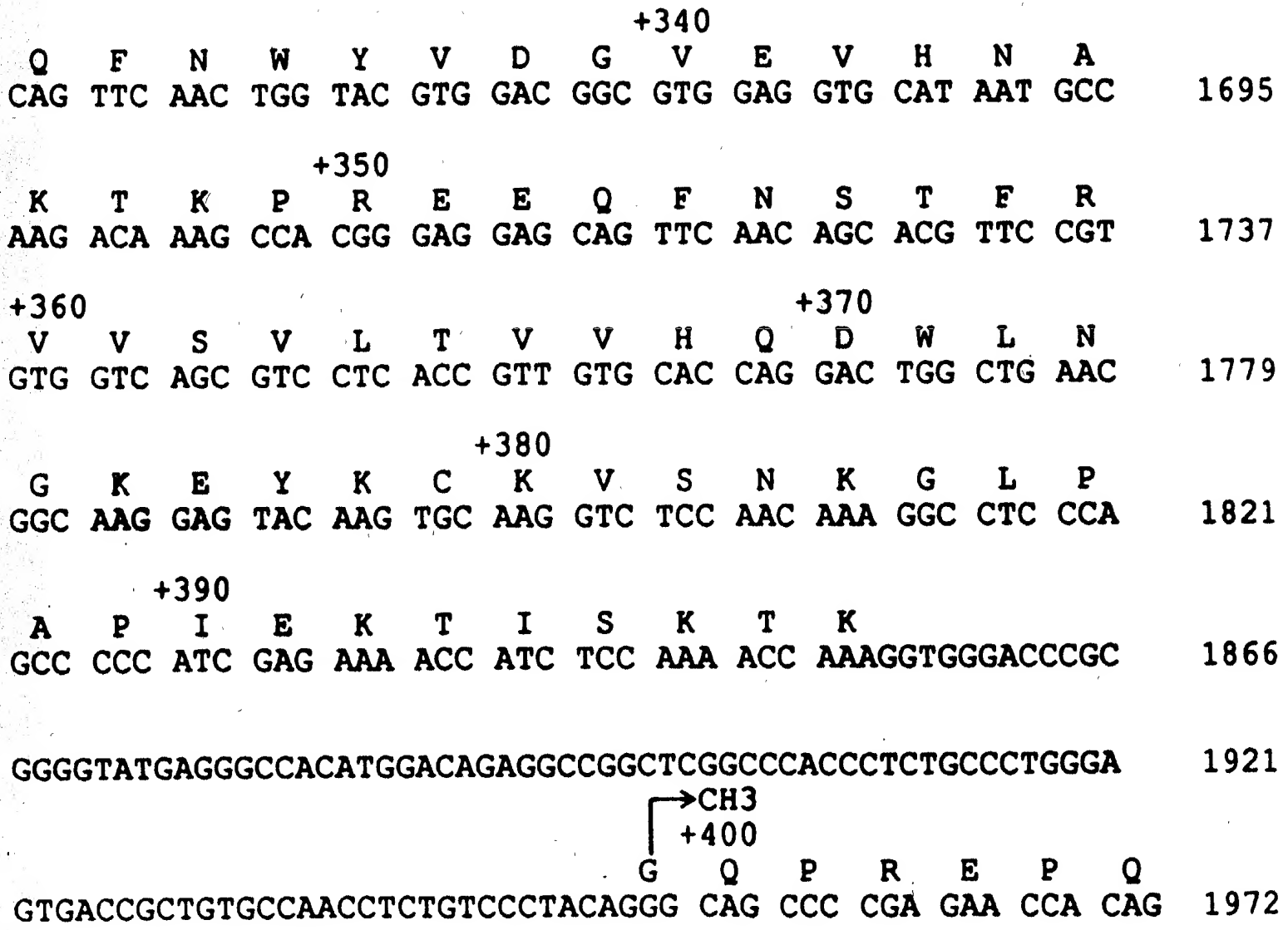

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                +410
 V   Y   T   L   P   P   S   R   E   E   M   T   K   N
GTG TAC ACC CTG CCC CCA TCC CGG GAG GAG ATG ACC AAG AAC     2014

+420                                    +430
 Q   V   S   L   T   C   L   V   K   G   F   Y   P   S
CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAC CCC AGC     2056

                        +440
 D   I   A   V   E   W   E   S   N   G   Q   P   E   N
GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC     2098

        +450                                    +460
 N   Y   K   T   T   P   P   M   L   D   S   D   G   S
AAC TAC AAG ACC ACA CCT CCC ATG CTG GAC TCC GAC GGC TCC     2140

                                +470
 F   F   L   Y   S   K   L   T   V   D   K   S   R   W
TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG     2182

                +480
 Q   Q   G   N   V   F   S   C   S   V   M   H   E   A
CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT     2224

+490                                    +500
 L   H   N   H   Y   T   Q   K   S   L   S   L   S   P
CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG     2266
```

```
 G   K  stop
GGT AAA TGAGTGCCACGGCCGGCAAGCCCCCGCTCCCCAGGCTCTCGGGGTCG     2319

CGTGAGGATGCTTGGCACGTACCCCGTGTACATACTTCCCAGGCACCCAGCATGG     2374

AAATAAAGCACCCAGCGCTGCCCTGGGCCCCTGCGAGACTGTGATGGTTCTTTCC     2429

GTGGGTCAGGCCGAGTCTGAGGCCTGAGTGGCATGAGGGAGGCAGAGTGGGTC...   2482
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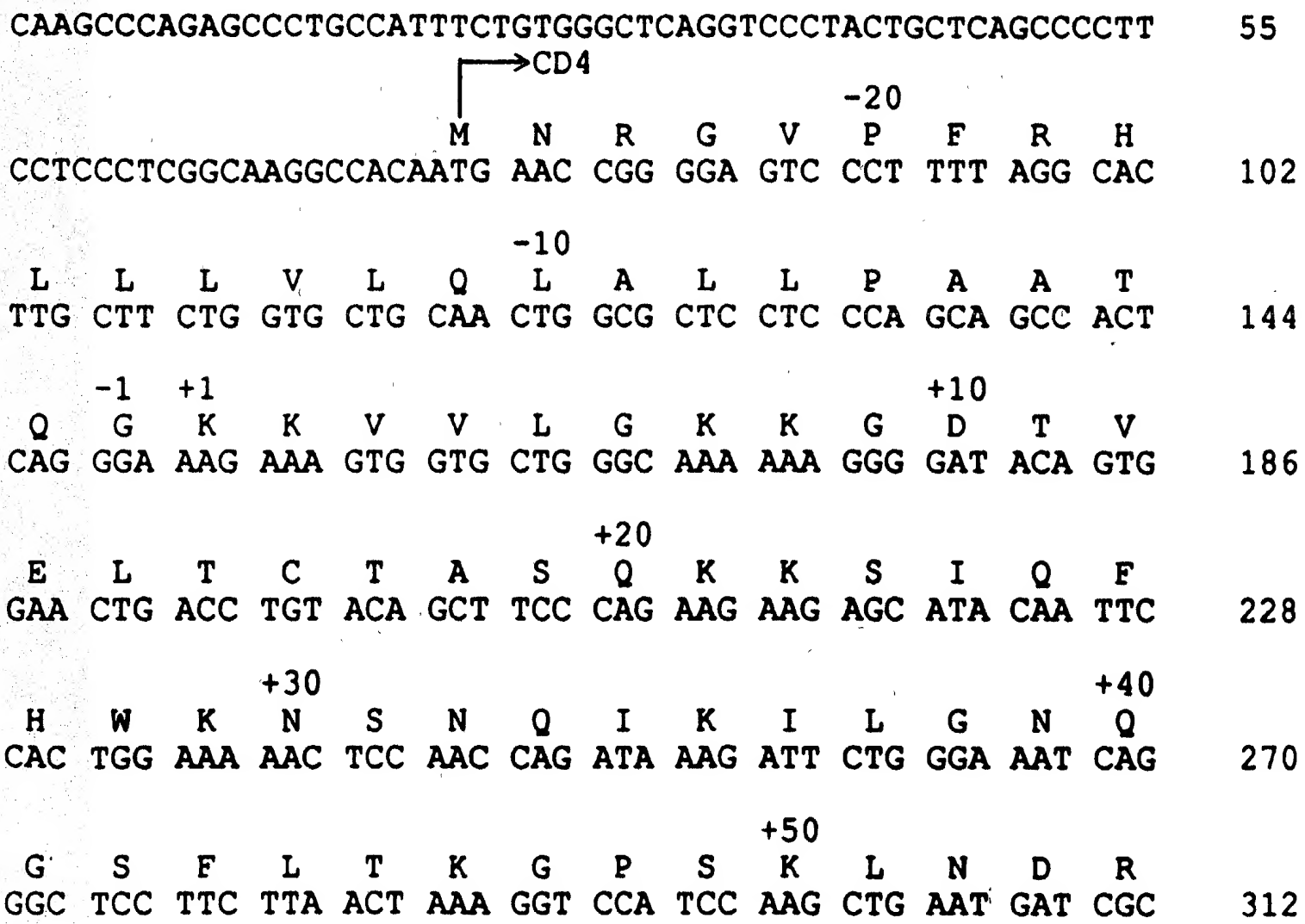
FIGURE 5



```
                         +60
 A   D   S   R   R   S   L   W   D   Q   G   N   F   P
GCT GAC TCA AGA AGA AGC CTT TGG GAC CAA GGA AAC TTC CCC    354

    +70                                 +80
 L   I   I   K   N   L   K   I   E   D   S   D   T   Y
CTG ATC ATC AAG AAT CTT AAG ATA GAA GAC TCA GAT ACT TAC    396

                             +90
 I   C   E   V   E   D   Q   K   E   E   V   Q   L   L
ATC TGT GAA GTG GAG GAC CAG AAG GAG GAG GTG CAA TTG CTA    438

           +100                                     +110
 V   F   G   L   T   A   N   S   D   T   H   L   L   Q
GTG TTC GGA TTG ACT GCC AAC TCT GAC ACC CAC CTG CTT CAG    480

                                     +120
 G   Q   S   L   T   L   T   L   E   S   P   P   G   S
GGG CAG AGC CTG ACC CTG ACC TTG GAG AGC CCC CCT GGT AGT    522

                     +130
 S   P   S   V   Q   C   R   S   P   R   G   K   N   I
AGC CCC TCA GTG CAA TGT AGG AGT CCA AGG GGT AAA AAC ATA    564
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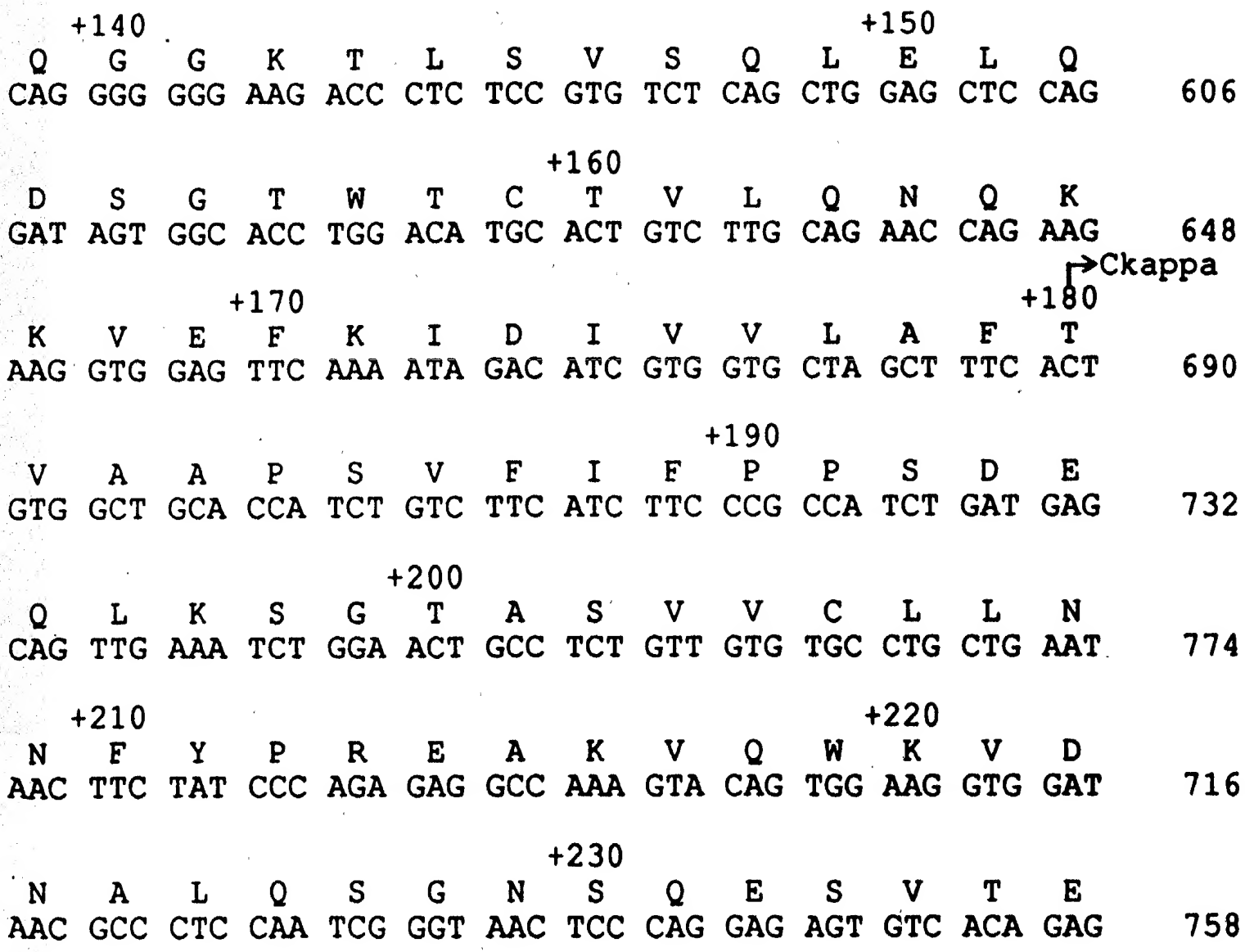

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            +240                                            +250
 Q   D   S   K   D   S   T   Y   S   L   S   S   T   L
CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC CTG      900

                                        +260
 T   L   S   K   A   D   Y   E   K   H   K   V   Y   A
ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC      942

                        +270
 C   E   V   T   H   Q   G   L   S   S   P   V   T   K
TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG      984

    +280
 S   F   N   R   G   E   C  stop
AGC TTC AAC AGG GGA GAG TGT TAG AGGGAGAAGTGCCCCCACCTGCTC  1032

CTCAGTTCCAGCCTGACCCCCTCCCATCCTTTGGCCTCTGACCCTTTTTCCACAGG  1088

GGACCTACCCCTATTGCGGTCCTCCAAGCTCATCTTTCACCTCACCCCCCTCCTCC  1144

TCCTT
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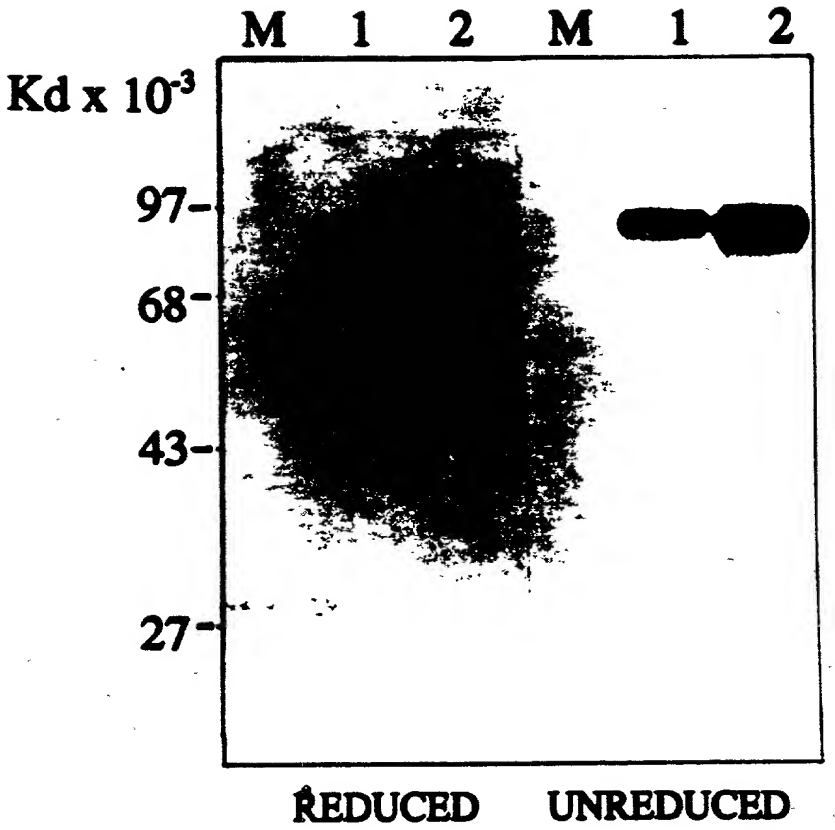
Figure 6



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RO/US 04 MAY 1992

07/960,440

Figure 7

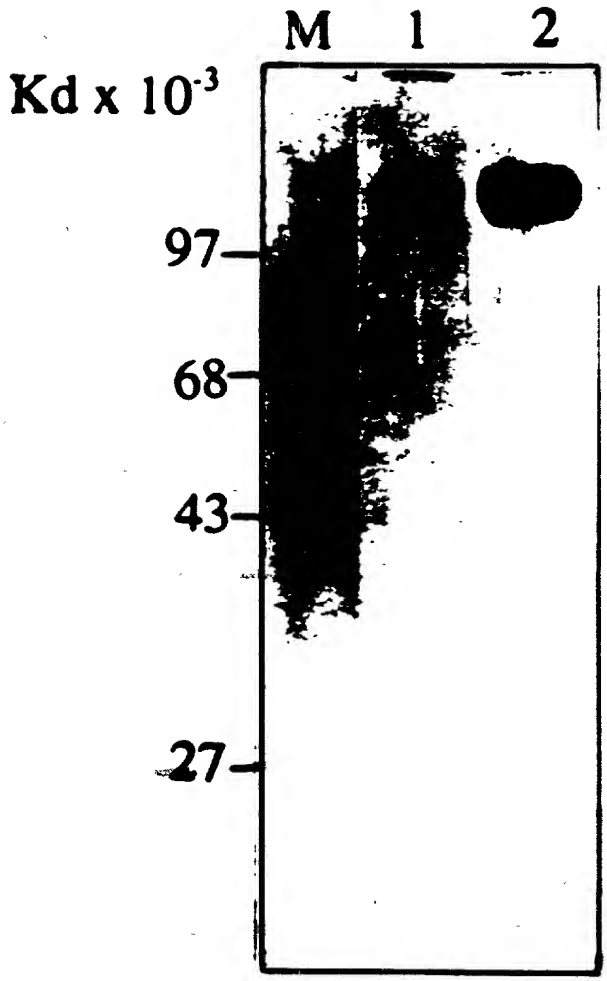


Figure 8

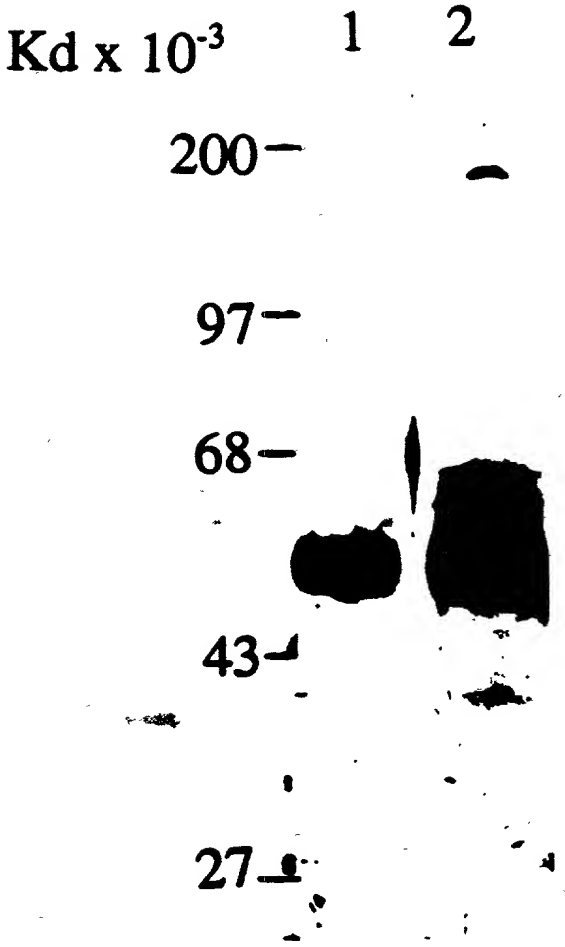


FIGURE 9

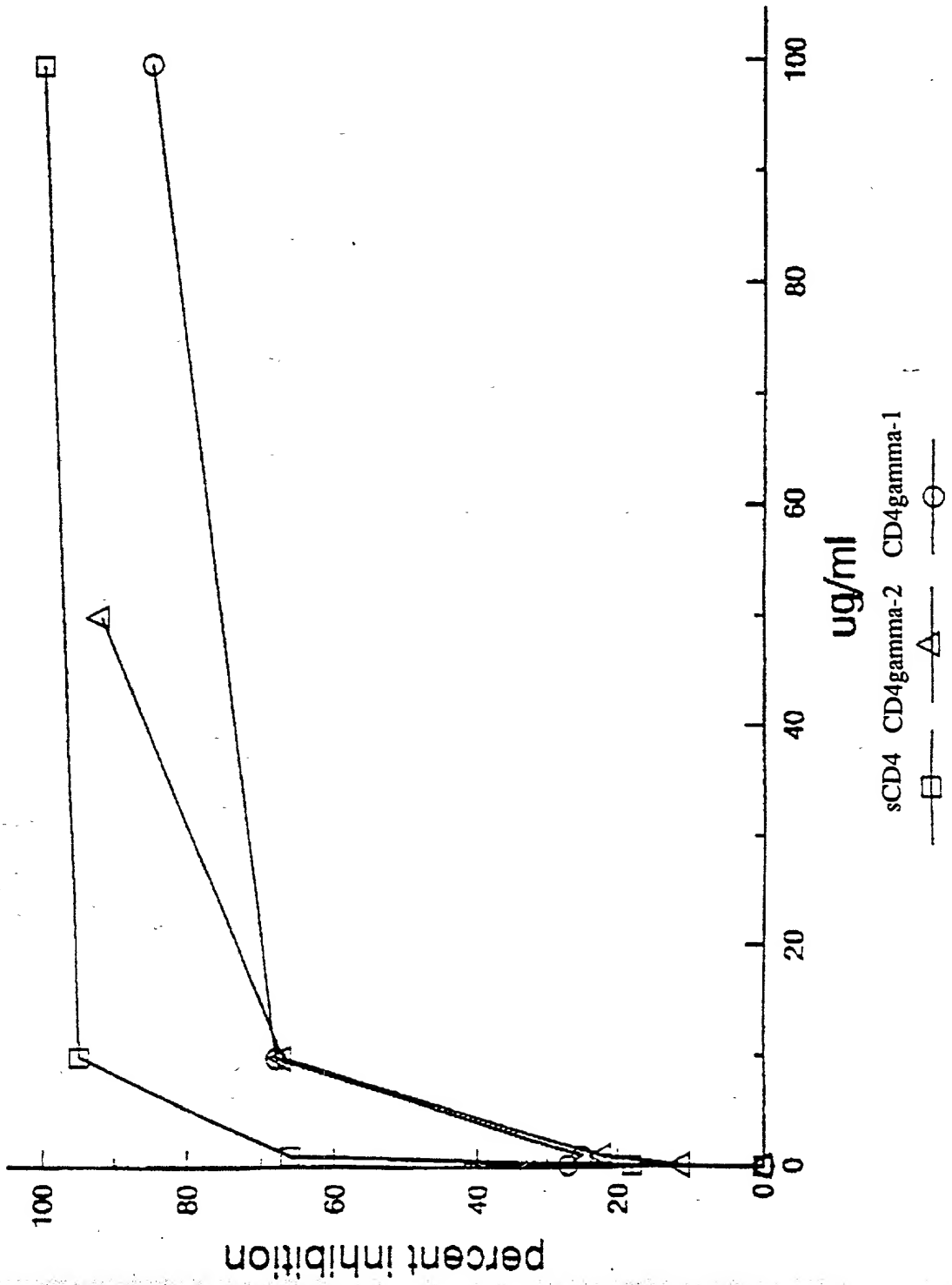

percent inhibition
0
20
40
60
80
100
0
20
40
60
80
100
ug/ml
sCD4
CD4gamma-2
CD4gamma-1

FIGURE 10

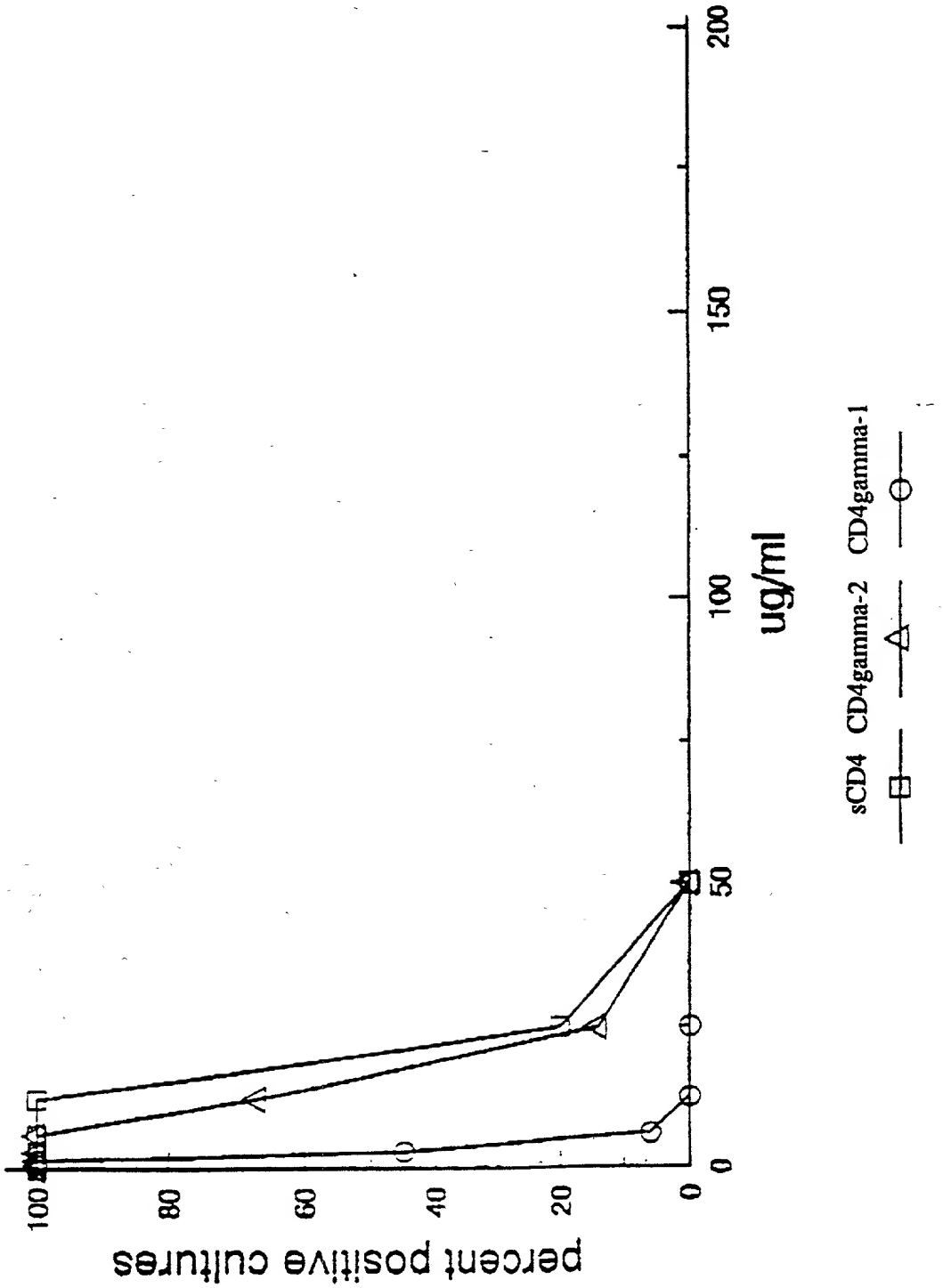

percent positive cultures
0
20
40
60
80
100
0
50
100
150
200
ug/ml
sCD4
CD4gamma-2
CD4gamma-1

Figure 11

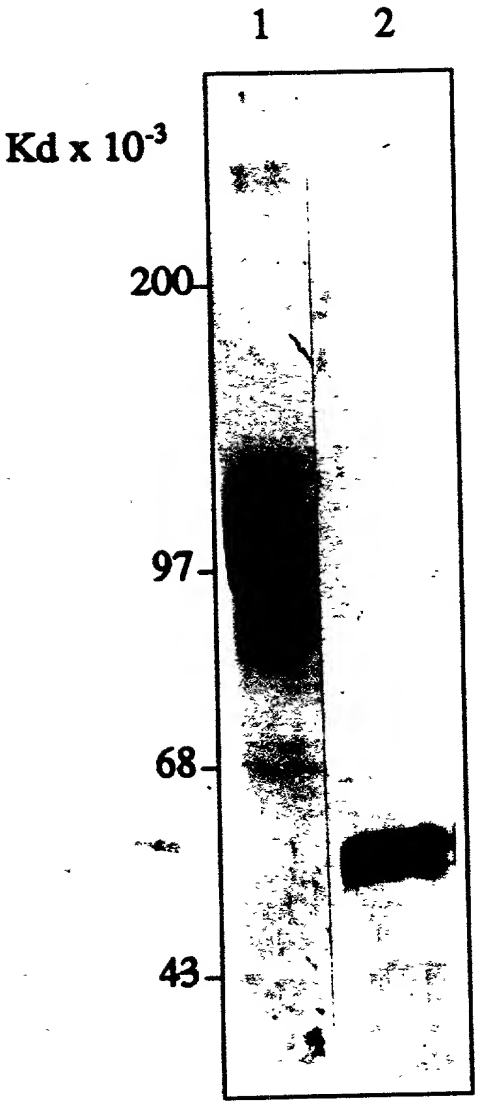
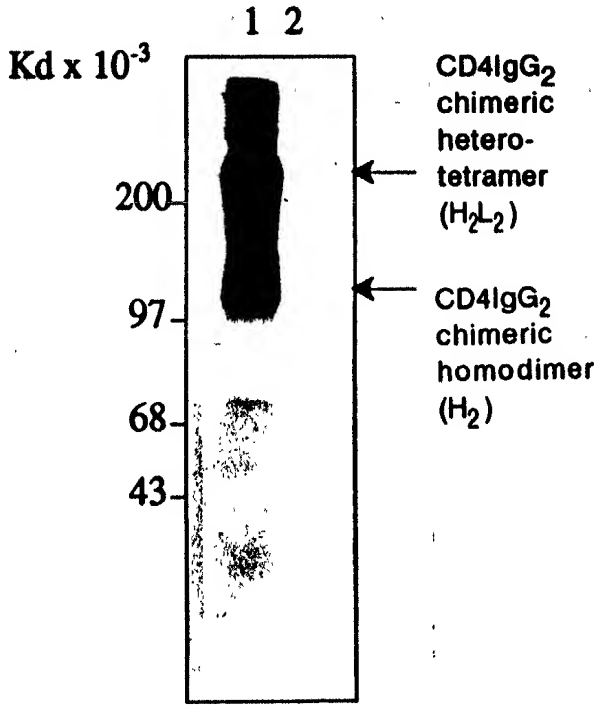
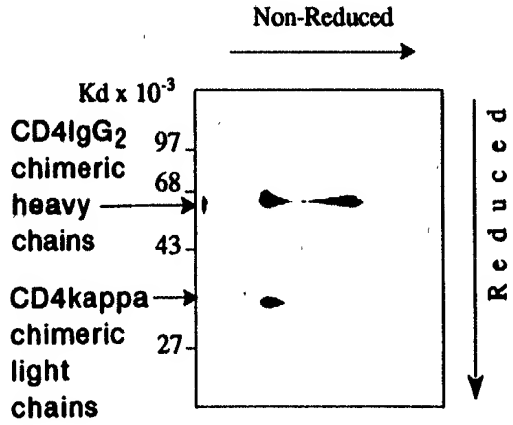


Figure 12A



Non-Reduced

Figure 12B



Applicant or Patentee: [redacted] Gary A. Beaudry and Paul J. Ma[redacted] Attorney's
Serial or Patent No.: [redacted]/US92/01143 Docket No.: 37690-II-PCT-US
Filed or Issued: 10 February 1992
Title of Invention or Patent: CD4-GAMMA2 AND CD4-IgG2 CHIMERAS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS UNDER 37 C.F.R. §1.9(f) AND §1.27(c) - SMALL BUSINESS CONCERN

I hereby declare that I am:

_____ the owner of the small business concern identified below.

__X__ an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Concern: Progenics Pharmaceuticals, Inc.

Address of Concern: Old Saw Mill River Road
Tarrytown, New York 10591

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 C.F.R. §121.3-18, reproduced in 37 C.F.R. §1.9(d), for purposes of paying reduced fees under 35 U.S.C. §41(a) and §41(b), in that the number of employees of the concern, including those of its affiliates, does not exceed five hundred (500) persons. For purposes of this verified statement, the number of employees of the business concern is the average number, over the previous fiscal year, of the persons employed by the business concern on a full-time, part-time, or temporary basis during each pay period of the fiscal year, and concerns are affiliates of each other when, either directly or indirectly, one concern controls or has power to control the other, or a third party or parties controls or has power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled

CD4-GAMMA2 AND CD4-IgG2 CHIMERAS

described in:

_____ the specification filed herewith
__X__ application serial no. PCT/US92/01143 filed 10 February 1992
_____ patent no. _____ issued _____

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below[a] and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 C.F.R. §1.9(c)*, any concern which could not qualify as a small business concern under 37 C.F.R. §1.9(d)* or as a nonprofit organization under 37 C.F.R. §1.9(e)*.

Name: N/A
Address: _____

____Individual ____ Small Business Concern ____ Nonprofit Organization

[a]NOTE: Separate verified statements are required for each named person, concern, or organization having rights to the invention averring to their status as small entities. 37 C.F.R. §1.27.

*See Reverse

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. 37 C.F.R. §1.28(b)*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Paul J. Maddon, M.D., Ph.D.
Title In Organization: Chairman and Scientific Director
Address: Old Saw Mill River Road
Tarrytown, New York 10591
Signature: Paul J. Maddon
Date Of Signature: Dec. 4, 1992

*See Reverse

Declaration and Power of Attorney

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

CD4-GAMMA2 AND CD4-IgG2 CHIMERAS

the specification of which
(check one)

____ is attached hereto.

__X__ was filed on 10 February 1992 as

Application Serial No. PCT/US92/01143

and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information of which I am aware which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s) Number	Country	Filing Date	Priority Claimed Yes	No
N/A				

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Sections 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	*Filing Date*	*Status*
U.S. Serial No. 653,684	February 8, 1991	Pending

And I hereby appoint

10

John P. White, Reg. No. 28,678; Thomas F. Moran, Reg. No. 16,579; Norman H. Zivin, Reg. No. 25,385; Ivan S. Kavrukov, Reg. No. 25,161; Christopher C. Dunham, Reg. No. 22,031; Thomas G. Carulli, Reg. No. 30,616; Robert D. Katz, Reg. No. 30,141; Peter J. Phillips, Reg. No. 29,691; Richard S. Milner, Reg. No. 33,970; and Robert J. Cobert Reg. No. 36,108.

and each of them, all c/o Cooper & Dunham of 30 Rockefeller Plaza, New York, New York 10112 (Tel. 212 977-9550), my attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International Applications which are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to

John P. White *Reg. No.* 28,678
Cooper & Dunham
30 Rockefeller Plaza
New York, N.Y. 10112
Tel. (212) 977-9550

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

10

Full name of sole or first joint inventor Gary A. Beaudry

Inventor's signature Gary A Beaudry

Citizenship United States of America *Date of signature* December 3, 1992

Residence 109 Inwood Avenue, Upper Montclair, New Jersey 07043 USA

Post Office Address Same as residence

NJ

2-90

Full name of joint inventor (if any) Paul J. Maddon

Inventor's signature Paul J. Maddon

Citizenship United States of America Date of signature Dec. 4, 1992

Residence 60 Haven Avenue, Apt. 25C, New York, New York 10032 USA NY

Post Office Address Same as residence

Full name of joint inventor (if any) ______

Inventor's signature ______

Citizenship ______ Date of signature ______

Residence ______

Post Office Address ______